Universidade de Aveiro Departamento de Biologia
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SARA RAQUEL BOAVENTURA RODRIGUES

Efeitos de compostos anticolinesterásicos em *Lepomis gibbosus*

Effects of anticholinesterase compounds on *Lepomis gibbosus*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – ramo Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Doutor Bruno Nunes (Professor auxiliar da Faculdade de Ciências da Saúde da Universidade Fernando Pessoa), da Doutora Sara Cristina Antunes (Estagiária de Pós-doutoramento do Departamento de Biologia e CESAM, Universidade de Aveiro) e do Doutor Bruno Castro (Investigador Auxiliar do Departamento de Biologia e CESAM, Universidade de Aveiro).

Dedico este trabalho aos meus pais e irmãos que comigo lutaram.

"A ciência é uma aventura de toda a raça humana para aprender a viver e talvez a amar o Universo onde se encontra. Ser uma parte dele é compreender, é conhecer-se a si próprio, é começar a sentir que existe dentro do Homem uma capacidade muito superior a que ele pensava ter e uma quantidade infinita de possibilidades humanas".

Isidor Isaac Rabi
(1898-1988)

o júri

presidente

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palavras-chave

Lepomis gibbosus, Monitorização ambiental, Acetilcolinesterase (AChE), Comportamento, Xenobióticos.

resumo

Nas últimas décadas, a investigação e sensibilização para as questões relacionadas com a exposição ambiental a xenobióticos tem aumentado, tendo-se vindo a conhecer a variedade de efeitos sobre os sistemas bióticos. Ao chegarem ao meio aquático, os xenobióticos podem afectar organismos não-alvo, colocando em risco o equilíbrio dos ecossistemas. Assim, os ensaios ecotoxicológicos têm sido utilizados como ferramentas de diagnóstico precoce (e.g. biomarcadores). A monitorização da inibição da acetilcolinesterase (AChE) em peixes tem sido amplamente utilizada em estudos ecotoxicológicos como um indicador de exposição a poluentes neurotóxicos. Contudo, o estabelecimento de ligação entre os diferentes níveis de organização biológica é uma questão importante na avaliação do impacto dos poluentes. Assim, a avaliação de respostas comportamentais deverá ser englobada em estudos ambientais, uma vez que o comportamento demonstra a capacidade de um indivíduo interagir directamente com o ambiente circundante.

Com base nestes pressupostos teóricos, a presente dissertação pretendeu gerar dados ecotoxicológicos de alguns compostos anticolinesterásicos, numa espécie não alvo, para futuras avaliações de risco e/ou monitorização ambiental para três classes de compostos: detergentes (SDS), pesticidas (clorfenvinfos) e fármacos (neostigmina e piridostigmina). Neste contexto, foi seleccionado o peixe *Lepomis gibbosus* (perca-sol) como organismo de estudo. Este apresenta-se como sendo um potencial candidato a organismo teste em ensaios ecotoxicológicos, por ser abundante, por ter requisitos simples de manutenção em laboratório e por não ser uma espécie ameaçada. Inicialmente, foi feita uma caracterização das colinesterases existentes na cabeça e músculo dorsal de *L. gibbosus*. De seguida, foram realizadas exposições *in vitro* e *in vivo* de modo a avaliar os efeitos do detergente aniónico SDS e do insecticida organofosforado (clorfenvinfos) na AChE de *L. gibbosus*. Por outro lado, foram ainda realizados ensaios *in vivo* de modo a avaliar o efeito dos fármacos neostigmina e piridostigmina na actividade da AChE (cabeça e músculo dorsal). Durante estas experiências foi ainda desenvolvido um ensaio comportamental de modo a avaliar possíveis alterações em *L. gibbosus* após exposição a estes xenobióticos.

Os resultados demonstraram que a acetilcolinesterase é a forma enzimática predominante na cabeça e músculo de *L. gibbosus*. A exposição ao clorfenvinfos revelou inibição da actividade da AChE *in vivo* e *in vitro*, contrariamente ao SDS - que não apresentou efeitos inibitórios. Relativamente aos fármacos, foram registados efeitos inibitórios na AChE, mais notórios no caso da cabeça e para a piridostigmina. Não obstante, não foram observadas alterações significativas nos parâmetros comportamentais, comparativamente aos organismos não expostos.

keywords

Lepomis gibbosus, Environmental monitoring, Acetylcholinesterase (AChE), Behavior, Xenobiotics.

abstract

In recent decades, research and awareness of issues related to environmental exposure to xenobiotics has increased. Upon their arrival in the aquatic environment, xenobiotics can affect non-target organisms, endangering the balance of ecosystems. Thus, ecotoxicological tests have been used as early diagnostic tools (e.g. biomarkers). Inhibition of acetylcholinesterase (AChE) activity in fish has been widely used in ecotoxicological studies as an indicator of exposure to neurotoxic pollutants. However, establishing a link between different levels of biological organization is an important issue when assessing the impact of pollutants. Thus, assessment of behavioral responses should be incorporated in environmental studies, because behavior demonstrates the ability of an individual to interact directly with the surrounding environment.

Based on these theoretical assumptions, this thesis sought to generate toxicological data for some anticholinesterasic compounds in a non-target species – for future risk assessments and / or environmental monitoring – for three classes of compounds, detergents (SDS), pesticides (chlorfenvinphos) and drugs (neostigmine and pyridostigmine). In this context, the fish *Lepomis gibbosus* (pumpkinseed) was selected as test-organism. It presents itself as a potential candidate as a test-organism in ecotoxicological tests because it is abundant, it has simple laboratory requirements, and it is not a threatened species. Initially, we performed a characterization of cholinesterases in the head and dorsal muscle of *L. gibbosus*. Then, we evaluated the effects of *in vitro* and *in vivo* exposures to the anionic detergent SDS and the organophosphate pesticide chlorfenvinphos on the AChE of *L. gibbosus*. *In vivo* exposures were also performed in order to evaluate the effects of the pharmaceutical drugs neostigmine and pyridostigmine on the activity of AChE (head and dorsal muscle). During these experiences, a behavioral trial was assayed for assessing possible changes in *L. gibbosu*s after exposure to these xenobiotics.

Results showed that acetylcholinesterase is the predominant enzymatic form in the head and muscle of *L. gibbosus*. Exposure to chlorfenvinphos showed inhibition of AChE activity both *in vivo* and *in vitro*, unlike SDS - which showed no inhibitory effects. For pharmaceutical drugs, inhibitory effects on AChE were recorded, particularly in the case of pyridostigmine, in the head of *L. gibbosus*. However, there were no concomitant significant changes in behavioral parameters of exposed animals, compared to untreated organisms.

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Capítulo I

Introdução Geral

1. Contaminação do ambiente aquático – presença de xenobióticos

O ambiente aquático tem uma enorme importância para os humanos, podendo ser fonte de água (para consumo e actividades antropogénicas), alimento e actividades lúdicas. No entanto, as descargas de resíduos industriais, deposição atmosférica e lixiviação dos solos tornam o ecossistema aquático no receptor final de contaminantes, incluindo diversas substâncias biologicamente activas, como milhares de compostos químicos orgânicos e inorgânicos sintéticos estanhos aos organismos (xenobióticos) (Newman *et al.,* 2001; Van der Oost *et al.,* 2003). Estes xenobióticos perturbam o funcionamento dos ecossistemas naturais, causando efeitos nefastos nos organismos, como o aumento da letalidade, muitas vezes levando à eliminação (extinção) das espécies mais sensíveis e à dominância das espécies mais resistentes e oportunistas (Bresler *et al.,* 1999). Assim, a intensificação de determinadas actividades (e.g. agricultura, indústria, urbanização), quando exercidas de forma descontrolada e sem um devido acompanhamento técnico e tecnológico, pode gerar ou libertar elementos e compostos perniciosos, sob várias formas (Bresler *et al.,* 1999; Ballesteros *et al.,* 2009). Ao atingirem o meio aquático e terrestre, os xenobióticos podem afectar organismos não-alvo, colocando em risco a saúde dos ecossistemas. Além disso, os xenobióticos são produzidos para actuar em vias metabólicas específicas, tais como receptores, canais iónicos e enzimas, os quais muitas vezes estão presentes também em organismos não alvo, causando diversos efeitos nocivos tais como a diminuição da capacidade reprodutiva, alimentar, crescimento ou de fuga aos predadores (Walker *et al.,* 2001; Van der oost, 2003).

As águas doces são o suporte de sistemas ecológicos complexos e específicos; dentre estes sistemas, são particularmente importantes os sistemas lacustres de pequena dimensão e reduzida profundidade (charcos, lagos, e lagoas), pois são os mais vulneráveis a alterações ambientais (Dugan, 1994). Das ameaças aos sistemas de água doce, destaca-se a poluição, mormente por material orgânico, que acarreta a acumulação de nutrientes e subsequente aumento de produtividade biológica de lagos e reservatórios, conhecida como eutrofização (Walker *et al.,* 2001).

Nas últimas décadas, a investigação e sensibilização para as questões relacionadas com a exposição ambiental a xenobióticos tem aumentado, tendo-se vindo a conhecer a variedade de efeitos sobre os sistemas bióticos. As consequências a longo prazo e as implicações para a espécie humana são também motivos de preocupação quando se considera a circulação global de substâncias químicas. Deste modo, a avaliação de efeitos sobre os ecossistemas aquáticos é uma tarefa importante, uma vez que estes sistemas são os locais mais afectados após contínuas descargas de contaminantes ambientais (Nunes *et al.,* 2005).

Contaminação com detergentes

Os compostos utilizados no fabrico de produtos higiénico-sanitários podem causar efeitos difusos, mas naturalmente perniciosos, nos processos fisiológicos de organismos expostos (Feng *et al.*, 2008). Assim, alguns destes compostos são lançados no ambiente aquático em elevadas quantidades, excedendo a capacidade de degradação natural do ambiente. Os detergentes modernos são biodegradáveis na água e são considerados como não acumuláveis no meio ambiente (Malcom *et al.*, 1995). No entanto, devido a uma entrada contínua, podem atingir concentrações relativamente elevadas em determinadas áreas (Feng *et al.*, 2008). Nesta situação encontram-se os detergentes aniónicos que entram na composição de muitos produtos para cuidados pessoais, como champôs, banhos de espuma, pastas de dentes e adjuvantes em preparações dermocosméticas e farmacêuticas (Sirisattha *et al.*, 2004). Um estudo de revisão efectuado por Csheráti *et al.* (2002) recolheu evidências que sustentam o envolvimento dos detergentes aniónicos em alterações biológicas, como a perturbação no funcionamento normal das vias bioquímicas e modificações estruturais, em várias espécies não alvo expostas por via ambiental. Assim, demonstrou-se que os detergentes exercem vários efeitos no ambiente, uma vez que se ligam a macromoléculas bioactivas, como proteínas com actividade enzimática. Os detergentes causam disrupções no funcionamento celular, uma vez que podem sofrer inserção em fragmentos celulares, como membranas fosfolipídicas (Csheráti *et al.*, 2002).

Dodecil sulfato de sódio (SDS) é um detergente aniónico bastante utilizado em produtos de uso doméstico e misturas industriais, produtos de limpeza e em cosméticos (Sirisattha *et al.*, 2004). SDS é considerado um dos detergentes mais comummente usado como surfactantes sintéticos, uma vez que é encontrado em champôs e sabonetes líquidos, espumas e géis de banho, e pastas de dentes (Sirisattha *et al.*, 2004; Nunes *et al.*, 2005; Feng *et al.*, 2008). As elevadas concentrações deste composto encontradas no ambiente, devem-se à sua entrada contínua, principalmente em locais com grande actividade antropogénica, podendo representar níveis consideráveis de contaminação (Nunes *et al.*, 2005; Feng *et al.*, 2008; Nunes *et al.*, 2008; Gonçalves *et al.*, 2010). Por exemplo, concentrações entre 0,2 e 10 mg L^{-1} de SDS foram encontradas em áreas de irrigação contaminadas com águas residuais (Dizer, 1990). Este composto tem sido altamente estudado pois aumenta a tensão superficial das monocamadas de fosfatidilcolina (Cserháti *et al.*, 2002), logo é de extrema importância determinar os seus efeitos sobre os processos celulares que envolvem fosfolípidos. Por outro lado, os efluentes provenientes de instalações de saúde correspondem a misturas complexas, frequentemente constituídos por grandes quantidades medicamentos, detergentes e desinfectantes (Kümmerer, 2001). Um estudo desenvolvido por Kümmerer (2001) demonstrou que a eficácia da remoção destes compostos em estações de tratamento de águas é reduzida devido à interacção entre os componentes das misturas complexas do esgoto, resultando

na libertação de grandes quantidades para o ambiente. Assim, estes compostos são geralmente estáveis, resistentes à biodegradação e, no caso dos detergentes, interferem com os processos normais de funcionamento microbiológico do qual depende o correcto funcionamento das ETARs.

Contaminação com pesticidas

A contaminação dos ecossistemas por pesticidas é preocupante, pois estes compostos são utilizados em larga escala para combater pragas que afectam a produção agrícola. No entanto, podem potencialmente afectar outras espécies, causando efeitos prejudiciais graves em organismos não alvos (Varó *et al.*, 2002; Van Dyk e Pletschke, 2011). Como frequentemente existem massas de água superficiais (rios, lagos, lagoas) junto de áreas agrícolas, é comum encontrarem-se resíduos de pesticidas nestes ecossistemas. O transporte destes resíduos, desde a área de aplicação até às massas de água, depende das suas propriedades físicas e químicas e ocorre geralmente através de processos de deriva por aspersão (transporte de partículas suspensas nas massas de ar corrente), escorrência à superfície do solo ou lixiviação (infiltração no solo) (Carter, 2000; Huber *et al.*, 2000; Pereira *et al.*, 2009). Estes contaminantes constituem frequentemente uma ameaça às comunidades aquáticas, e o estudo dos seus efeitos em organismos que aí vivem permite obter dados que facilitam a compreensão da extensão dos danos que podem ocorrer nos ecossistemas aquáticos (Arufe *et al.*, 2007; Pereira *et al.*, 2009).

Os resíduos de pesticidas têm sido detectados em diversas matrizes aquáticas (Cerejeira *et al.*, 2003; Guest *et al.*, 2006; Wilson e Foos, 2006; Pereira *et al.*, 2009), em concentrações que frequentemente ultrapassam os máximos permitidos pela legislação aplicável (e.g. CE, 1998). A presença de um xenobiótico num compartimento ambiental não indica, por si só, que este último sofra efeitos adversos. Em oposição, as baixas concentrações ambientais de xenobióticos não são necessariamente inócuas, uma vez que podem desencadear um conjunto de efeitos crónicos, quer pela sua acção individual ou combinada, que podem acabar por comprometer a sustentabilidade das populações naturais (Peakall, 1992).

Os pesticidas são, frequentemente, muito persistentes com semi-vidas de décadas e são transportados para longas distâncias através de lixiviação e escorrências, alcançando o meio aquático (Van Dyk e Pletschke, 2011). Assim, a poluição do ambiente por pesticidas, e particularmente da água, tem-se tornado um problema mundial (Ongley, 1996). O uso de carbamatos e organofosforados tem crescido progressivamente em alternativa aos organoclorados, cuja aplicação já foi proibida em muitos países (Ssebugere *et al.*, 2010). Os organoclorados são pesticidas potencialmente perigosos, persistentes no ambiente, com tendência a acumular-se nas cadeias alimentares. Estes pesticidas foram classificados, pela Organização Mundial de Saúde, como sendo "possíveis compostos cancerígenos para os humanos". Em oposição, os pesticidas

organofosforados (OP) e carbamatos (CB) são comummente utilizados nas práticas agrícolas como insecticidas, porque são, em geral, mais biodegradáveis e com menor persistência no meio ambiente do que os pesticidas organoclorados (Varó *et al.*, 2002). No entanto, são motivo de preocupação ecológica, uma vez que são tóxicos para espécies não-alvo em baixas concentrações. A toxicidade destes pesticidas é principalmente devido à inibição da enzima acetilcolinesterase (AChE), por serem compostos neurotóxicos (Fukuto, 1990).

Cerejeira *et al.* (2003) realizaram vários estudos (de 1983 a 1999) em algumas bacias hidrográficas localizadas nas proximidades das principais áreas agrícolas em Portugal e detectaram diversos insecticidas e herbicidas em amostras de água, incluindo clorfenvinfos (em concentrações entre 0,02 e 31,6 μgL^{-1}). Por outro lado, a União Europeia estabeleceu um novo quadro de acção comunitária no domínio da política da água (EC, 2000), definindo uma lista de substâncias prioritárias, na qual se inclui o clorfenvinfos (EC, 2001). A organização Mundial de Saúde (World Health Organization, WHO, 2004) classificou o clorfenvinfos, adicionalmente, como altamente perigoso devido às suas propriedades biologicamente activas. O pesticida clorfenvinfos, de forma molecular 2-cloro-1-(2,4-diclorofenil) vinil dietil fosfato, é um insecticida organofosforado sintético, e é usado para controlar pragas de insectos - como moscas e pulgas - e ácaros (ATSDR, 1997). Pode ser encontrado no solo, águas subterrâneas e em águas superficiais (rios e lagos) (ATSDR, 1997), tendo uma mobilidade média no ambiente terrestre ($\log K_{OC} = 2.47$) e acumulação moderada em organismos aquáticos (FAO, 2000). Serrano *et al.* (1997) estudaram a capacidade de acumulação do clorfenvinfos em mexilhão (*Mytilus galloprovincialis*), e determinaram que o factor de bioconcentração (BCF) era 255±78 L kg^{-1}. O tempo de semi-vida por hidrólise do clorfenvinfos, quando em meio aquoso, foi estimado em 4 dias a pH = 6 e cerca de 16,67 dias a pH = 6-8, a 20°C. Sismeiro-Vivas *et al.* (2007) estudaram os efeitos do clorfenvinfos em *Gambusia holbrooki*. Este estudo revelou que este insecticida se apresenta como potencialmente perigoso para os organismos aquáticos não-alvo, uma vez que foram detectadas alterações comportamentais e enzimáticas, nomeadamente na actividade da acetilcolinesterase, após exposição.

Contaminação com Fármacos

Os compostos de utilização farmacêutica, humana ou veterinária, reúnem uma série de características que fazem com que o estudo dos seus efeitos ambientais seja fundamental. Os fármacos são absorvidos pelo organismo e estão sujeitos a reacções metabólicas e, uma quantidade significativa dessas substâncias, tanto a original como os seus metabolitos, são excretadas por seres humanos e seus animais (de companhia ou produção agropecuária) por intermédio da urina e das fezes. Deste modo, o destino mais comum dos fármacos e seus metabolitos são as águas residuais. Vários estudos já efectuados demonstraram que as estações de tratamento de águas residuais não

são eficazes, e muitos fármacos passam quase inalterados (Winkler *et al.,* 2001; Petrović *et al.,* 2003), justificando a sua presença no compartimento aquático.

As águas residuais representam a principal via de entrada de resíduos de medicamentos nos ecossistemas aquáticos (Petrović *et al.,* 2003). Apesar dos esforços em termos de eliminação de nutrientes e contaminação microbiana, tal facto não foi observado relativamente a compostos sintéticos, como os medicamentos, produtos de higiene pessoal e seus resíduos (Ternes, 1998).

Os medicamentos são constituídos por substâncias biologicamente activas (Nunes *et al.,* 2005; Nunes *et al*., 2008), distinguindo-os da maioria dos compostos sintetizados pelos humanos. São compostos desenhados e obtidos quimicamente com o propósito de alterarem funções biológicas (vias metabólicas, receptores ou biomoléculas), e são utilizados na medicina veterinária, na saúde humana, na agricultura e aquacultura. São maioritariamente lipofílicos, sendo mais facilmente absorvidos pois conseguem atravessar membranas biológicas (Nunes *et al*., 2008). São persistentes e bioacumuláveis, pelo que podem exercer a sua actividade biológica. Apresentam baixos índices de degradação, podendo actuar por longos períodos de tempo, antes de serem excretados pelo organismo. O contínuo processo de libertação de fármacos para o ambiente resulta na sua presença sistemática, principalmente no ambiente aquático (Gonçalves *et al*., 2010). Deste modo, os compostos de utilização farmacêutica caracterizam-se por uso contínuo e indiscriminado, e por uma elevada actividade biológica no ambiente (Halling-Sørensen *et al*., 1998; Daughton e Ternes, 1999; Jones *et al*., 2002; Miao *et al*., 2002).

O consumo de fármacos tem aumentado ao longo do tempo em parte acompanhando o aumento do número de doenças tratáveis com recurso a estes, contribuindo para um aumento da longevidade. Segundo o relatório estatístico anual do INFARMED (Infarmed, 2009) o consumo de medicamentos em Portugal, entre 2005 e 2009, acompanha esta tendência de aumento. O uso indiscriminado de fármacos de venda livre ou sem prescrição médica, as drogas de abuso e também resíduos de medicamentos veterinários resultam inevitavelmente na sua libertação para o meio ambiente (Daughton e Ternes, 1999). Assim, a quantidade de substâncias com potencial acção farmacológica no meio ambiente poderá estar subestimada. A presença destes compostos no meio aquático interfere significativamente na fisiologia, no metabolismo e no comportamento das espécies expostas. É importante ainda realçar o facto de os fármacos causarem efeitos tóxicos sinérgicos na presença de outros compostos (Cleuvers, 2003).

Algumas das substâncias que são utilizadas na terapêutica humana exibem propriedades que lhes possibilitam a interferência com processos-chave, não exclusivos dos Humanos, como a neurotransmissão. A transmissão neuromuscular depende essencialmente da libertação de acetilcolina e da funcionalidade dos respectivos receptores a nível pós sináptico. Esta transmissão pode ser afectada, quer pela diminuição da disponibilidade de acetilcolina, quer disfunção dos

receptores, ou ambas (Ceremuga *et al*., 2002; Infarmed, 2010). Os compostos neostigmina e piridostigmina são inibidores das colinesterases, e têm sido extremamente utilizados, ao longo das últimas cinco décadas, no tratamento de desordens características da doença auto-imune miastenia gravis, síndrome miasténica congénita não auto-imune, glaucomas, e têm adicionalmente sido usados na protecção contra compostos organofosforados (Pope *et al*., 2005; Yu *et al*., 2010). Estes inibidores da acetilcolinesterase aumentam o tempo de permanência da acetilcolina na sinapse. Devido a este mecanismo de acção, estes medicamentos também são úteis na reversão do bloqueio muscular induzido por relaxantes musculares não-despolarizantes usados em anestesia (Infarmed, 2010). Estes compostos são altamente solúveis em água (Yu *et al*., 2010). Quando libertados para o meio ambiente, estes compostos irão exercer efeitos fisiológicos em organismos não-alvo, nomeadamente os peixes. Alguns estudos já foram efectuados, de forma a avaliar os efeitos após exposição destes compostos (brometos de neostigmina e piridostigmina) em mamíferos, nomeadamente ratos (Kempen *et al*., 1999; Joosen e Helden, 2007;) e humanos (Mirakhur *et al*., 1982; Ceremuga *et al*., 2002; Yu *et al*., 2010; Zimerman *et al*., 2010), os quais confirmam as suas propriedades anticolinesterásicas. Contudo, em toda a pesquisa não se conhecem efeitos destes, em organismos não-alvo, nomeadamente em peixes.

A miastenia gravis (MG) é uma doença neurológica auto-imune, que afecta o elemento pós-sináptico da junção neuromuscular. A maioria dos casos (85%) está associada a um anticorpo IgG, que actua contra os receptores nicotínicos de acetilcolina pós-sinápticos, reduzindo o número de receptores eficazes a um terço dos níveis normais (Gold e Schneider-Gold, 2008; Argov, 2009). Os problemas na transmissão neuromuscular, juntamente com a diminuição da formação de potenciais de acção pós-sinápticos, resultam na diminuição da contracção muscular, levando a características clínicas clássicas, como fraqueza muscular e cansaço (Conti-Fine *et al*., 2006). Estes agentes anticolinesterásicos, como neostigmina e piridostigmina, inibem a acção hidrolítica da enzima acetilcolinesterase. Deste modo, a possibilidade de neurotransmissor se ligar às placas motoras é mais intensa e ocorre durante um período de tempo mais longo, o que melhora a função muscular, antes debilitada (Ceremuga *et al*., 2002).

2. Ecotoxicologia e Bioensaios

O termo *Ecotoxicologia* foi descrito por René Truhaut em 1969, que o definiu como sendo "*o ramo da toxicologia preocupado com o estudo de efeitos tóxicos causados por compostos naturais ou sintéticos, sobre os organismos vivos constituintes dos ecossistemas, incluindo humanos, num contexto integrado"* (Truhaut, 1977). Mais tarde, a Ecotoxicologia foi definida como "*o estudo dos efeitos nefastos das substâncias químicas nos ecossistemas" (*Walker *et al*., 2001). Uma das primeiras grandes preocupações em ecotoxicologia foi a padronização das ferramentas a usar,

nomeadamente os bioensaios de toxicidade. Que critérios definir para seleccionar os organismos de teste? Como reduzir a variabilidade das respostas? Deste modo, tornou-se necessária a padronização dos bioensaios de toxicidade e o estabelecimento de parâmetros de comparação entre estes (Cairns e Pratt, 1989; Soares e Calow, 1993).

Ao longo das últimas décadas, os testes de toxicidade têm vindo a aumentar de complexidade, surgindo novos ensaios, novos parâmetros indicativos de toxicidade, bem como incorporadas novas espécies de peixes, moluscos e crustáceos (Mora *et al.*, 1999; Nunes *et al.*, 2005; Sismeiro-Vivas *et al.*, 2007; Bervoets *et al.*, 2009). No entanto, a sua utilidade e os seus objectivos não se alteraram: gerar informação que possa ser utilizada na avaliação de risco, monitorização e gestão ambiental (Buikema *et al*., 1982). Os testes de toxicidade são procedimentos nos quais as respostas dos organismos são usadas para detectar e medir os efeitos de uma ou mais substâncias, resíduos, ou factores ambientais, sozinhos ou em combinação, durante um determinado tempo. Com estas respostas, pode-se estimar, através de métodos estatísticos, a concentração dessas substâncias, que poderão causar toxicidade aos organismos representantes do ambiente receptor (Walker *et al*., 2001).

Os ensaios ecotoxicológicos têm sido utilizados, com sucesso, como ferramentas de diagnóstico precoce, permitindo regular o uso de algumas substâncias. A procura de novas técnicas do ponto de vista científico, tem sido considerada nos últimos anos, destacando-se a importância da relevância ecológica nos ensaios de toxicidade e, assim abordando novos parâmetros, que permitam obter respostas mais sensíveis, representando sinais de alerta precoce (Scott and Sloman, 2004). A incorporação de espécies residentes em ensaios de toxicidade é uma das formas de tornar os ensaios mais específicos para o local em estudo e, de modo geral, mais relevantes do ponto de vista ecológico. As respostas das espécies-padrão (*e.g.*, *Daphnia magna*) nem sempre reflectem a sensibilidade das espécies locais que se pretende avaliar ou proteger (Buikema *et al*., 1982).

A toxicidade é a resposta de um organismo a uma dose de determinado composto, que é mantida acima de uma concentração limite após um período de exposição. A resposta biológica é a soma de todas as alterações a que o organismo é submetido, bem como a capacidade de compensação desse organismo (Walker *et al*., 2001). Contudo, a avaliação da toxicidade deve ser feita utilizando parâmetros sensíveis e, se possível, que permitam detectar precocemente a exposição a contaminantes. A relação entre a sensibilidade e a relevância ecológica das respostas utilizadas em Ecotoxicologia está directamente relacionado com o encadeamento dos diversos níveis de organização biológica (Burton, 1991). As respostas sub-individuais aos contaminantes, por exemplo, são muito sensíveis e passíveis de serem quantificadas após um curto período de exposição. Assim, o seu significado ecológico é mais elevado quando se estabelecem relações entre

as alterações sub-individuais e os efeitos em níveis de organizacionais superiores (Burton, 1991; Peakall, 1992).

Os ensaios de toxicidade podem ser classificados segundo os diversos efeitos que os organismos venham a apresentar durante o tempo de exposição (Walker *et al.*, 2001). Os testes de toxicidade aguda proporcionam rápidas respostas na estimativa dos efeitos letais de um agente tóxico sobre os organismos, caracterizam-se pelo curto tempo de exposição (24 a 96 horas) a concentrações geralmente elevadas de determinada substância química. Nos testes de toxicidade crónica o tempo de exposição envolve períodos mais longos com concentrações sub-letais. Assim, o impacto dos poluentes sobre os organismos aquáticos pode ser estimado e monitorizado através de testes de toxicidade em condições de laboratório. A escolha dos parâmetros a avaliar depende do tipo de estudo que se quer desenvolver.

Biomarcadores – Acetilcolinesterase

Segundo Timbrell (1998) e Van der Oost *et al*. (2003), os biomarcadores podem ser divididos em três classes: 1) *biomarcadores de exposição* - abrangem a detecção e quantificação de um composto exógeno, dos seus metabolitos ou da interacção entre este e moléculas ou células alvo, sendo medidos num compartimento do organismo; 2) *biomarcadores de efeito* - incluem alterações bioquímicas e fisiológicas, sendo mensuráveis nos tecidos ou fluídos corporais e reconhecidamente associadas a possíveis desequilíbrios, e 3) *biomarcadores de susceptibilidade* - indicam a capacidade inerente ou adquirida de um organismo alterar a susceptibilidade a uma exposição, envolvendo nomeadamente factores genéticos. O autor Peakall (1992) seguiu uma perspectiva fisiológica, definindo uma divisão em *biomarcadores de neurotoxicidade* (*e.g.*, a actividade da acetilcolinesterase), de *stress oxidativo* (*e.g.*, a enzima superóxido dismutase), de *imunotoxicidade* (*e.g.*, concentração de imunoglobulinas), de *histopatologia* (*e.g.*, danos tecidulares, necrose), de *biotransformação* (*e.g.*, a indução de enzimas da fase I e II de biotransformação), de *genotoxicidade* (*e.g.*, a quantificação de quebras no DNA), de *disrupção endócrina* (*e.g.*, níveis de estrogénios), de *alterações em proteínas reguladoras* (*e.g.*, níveis de metalotioneínas, proteínas de stress ou "heat-shock proteins", algumas hormonas), do *metabolismo energético* (*e.g.*, o teor de reservas lipídicas ou de glicogénio), entre outros.

Os ensaios ecotoxicológicos permitem uma monitorização regular, sustentada pela avaliação dos efeitos biológicos causados por xenobióticos, recorrendo frequentemente a um leque adequado de respostas biológicas (biomarcadores). Estas respostas podem incluir alterações a nível bioquímico, fisiológico, celular, morfológico ou comportamental, permitindo assim um conhecimento mais aprofundado dos processos biológicos associados à acção dos contaminantes (Peakall, 1992; Timbrell, 1998; Van der Oost *et al.*, 2003). Num contexto ambiental, os

biomarcadores são considerados ferramentas altamente informativas no que respeita à detecção da exposição a contaminantes ou dos respectivos efeitos. A utilização de biomarcadores oferece ainda a possibilidade de obter um entendimento mecanístico ou de causa-efeito dos processos biológicos. Esta avaliação pode ser utilizada como sistema de alerta que permita prever e evitar efeitos tóxicos significativos, antecipando alterações nos níveis mais elevados de organização biológica (Ballesteros *et al.,* 2009).

Os biomarcadores são considerados ferramentas sensíveis, podendo ser quantificados através de procedimentos simples e padronizados (Van der Oost *et al.*, 2003). A sua versatilidade como ferramentas de análise ambiental pode ser demonstrada por vários estudos efectuados, em organismos distintos (peixes, mamíferos, moluscos, plantas, crustáceos e insectos), nos quais podem ser monitorizados parâmetros específicos, consoante o propósito do estudo em causa (e.g. Mora *et al.,* 1999; Nunes *et al.,* 2005; Nunes *et al.,* 2006; Leticia e Gerardo, 2008; Antunes *et al*., 2010; Pathiratne *et al.,* 2010). A quantificação de biomarcadores tem sido amplamente utilizada tanto *in vivo* como *in vitro* para a avaliação dos efeitos que os xenobióticos apresentam para os organismos (Binelli *et al.,* 2006). Apesar das respostas medidas a níveis organizacionais elementares poderem não ter reflexo directo ao nível da população ou comunidade, podem funcionar como um instrumento de vigilância ambiental que permite identificar e prever o risco a esses níveis.

Ao longo dos anos, as colinesterases (ChEs) têm sido utilizadas como potenciais biomarcadores para a monitorização da contaminação do ambiente por vários compostos, principalmente pesticidas organofosforados (OP) e carbamatos (CB) (Mora *et al.,* 1999; Fulton e Key, 2001; Binelli *et al.,* 2006; Feng *et al*., 2008; Ballesteros *et al*., 2009; Tilton *et al*., 2011), metais pesados (Garcia *et al.,* 2000; Nunes *et al.,* 2003; Vieira *et al.,* 2009), efluentes de minas (Castro *et al.,* 2004), resíduos de celulose e detergentes (Nunes *et al.,* 2005; Feng *et al.,* 2008; Li, 2008), fármacos (Nunes *et al.,* 2006; Gonçalves *et al.,* 2010). As colinesterases (ChEs) pertencem à família de enzimas designadas de esterases, as quais têm a capacidade de hidrolisar ésteres carboxílicos (Gonçalves *et al.,* 2010). As ChEs podem ser distinguidas de outras esterases, uma vez que apresentam preferência para a hidrólise de ésteres de colina, em vez de outros ésteres carboxílicos, e são inibidas pela fisostigmina (eserina) em concentrações na faixa de 10^{-5} M (Garcia *et al.,* 2000; Nunes *et al.,* 2003). Os vertebrados possuem dois tipos de ChEs: acetilcolinesterase (AChE, EC 3.1.1.7) e butirilcolinesterase ou pseudocolinesterase (BChE, EC 3.1.1.8), que diferem na sua especificidade de substrato e de inibidor (Mora *et al.,* 1999; Sturm *et al.,* 1999; Monteiro *et al.,* 2005; Leticia e Gerardo, 2008). A acetilcolinesterase (AChE), também conhecida como colinesterase verdadeira ou colinesterase específica, apresenta um papel fundamental na regulação da transmissão nervosa (Xuereb *et al.,* 2009; Gonçalves *et al.,* 2010). A principal função da AChE

é catalisar a hidrólise da acetilcolina em ácido acético e colina em locais sinápticos colinérgicos (Garcia *et al.*, 2000; Nunes *et al.*, 2003; Leticia e Gerardo, 2008). Na verdade, a AChE é responsável pela degradação hidrolítica da acetilcolina, que é o principal neurotransmissor nos sistemas sensoriais e neuromusculares na maioria das espécies animais (Xuereb *et al.*, 2009).

A monitorização da inibição da enzima colinesterase (ChE) em peixes tem sido amplamente utilizada em ecossistemas aquáticos como um indicador de exposição a poluentes neurotóxicos e consequentes efeitos fisiológicos (Nunes *et al.*, 2003; Sismeiro-Vivas *et al.*, 2007). A inibição da AChE provoca uma acumulação de acetilcolina nas sinapses nervosas e interrupção da função nervosa, levando à super-estimulação do sistema nervoso central e periférico, resultando em efeitos neurotóxicos deletérios nos organismos (Nunes *et al.*, 2003; Xuereb *et al.*, 2009; Gonçalves *et al.*, 2010). Diferentes formas colinesterásicas apresentam sobreposição de capacidades hidrolíticas (Nunes *et al.*, 2003). Na monitorização ambiental, é fundamental caracterizar a forma enzimática existente nos organismos expostos, de modo a saber o intervalo normal de actividade em indivíduos não-expostos (Garcia *et al.*, 2000; Varó *et al.*, 2002).

Comportamento

Apesar do reconhecido valor dos biomarcadores como ferramentas de alerta precoce, a importância de alguns estudos ambientais com base em biomarcadores tem sido questionada, principalmente devido ao facto de que as alterações induzidas a nível sub-individual não representam necessariamente efeitos deletérios em níveis superiores de organização biológica (Correia *et al.*, 2007; Vieira *et al.*, 2009). De acordo com Vasseur e Cossu-Leguille (2006), o estabelecimento de ligações entre os diferentes níveis de organização biológica é uma questão importante quando se considera o impacto dos poluentes químicos, pois a toxicidade é induzida, em primeiro lugar, a nível sub-individual e, em seguida, a nível individual, antes que as populações sejam afectadas. Portanto, usando os parâmetros a nível sub-individual, assim chamados de biomarcadores, os efeitos prejudiciais podem ser previstos precocemente (Gravato e Guilhermino, 2009).

Considerando o papel determinante da AChE na neurotransmissão, é provável que a inibição da actividade de AChE encontrada em peixes expostos a agentes neurotóxicos contribua para alterações comportamentais. Segundo vários autores, a actividade da AChE está significativa e positivamente correlacionada com alterações comportamentais (Scott e Sloman, 2004; Castro *et al*., 2009; Vieira *et al*, 2009; Xuereb *et al*, 2009). Assim, quando ocorre inibição da AChE, devido a exposição a agentes neurotóxicos, alterações significativas no comportamento (e.g. alimentação, locomoção) poderão ser observadas.

Embora a AChE desempenhe uma função essencial para a actividade do organismo, poucos estudos têm relacionado a inibição da AChE com parâmetros comportamentais que podem afectar a capacidade do organismo, causando alterações a níveis de organização biológicos mais elevados (Xuereb *et al*., 2009). O comportamento representa a capacidade de um indivíduo lidar directamente com o ambiente circundante e, ulteriormente, para se reproduzir e sobreviver (Engenheiro *et al*., 2005), pois relaciona as funções fisiológicas com processos ecológicos (Scott e Sloman, 2004). Portanto, tem sido dada importância ao estudo dos efeitos de tóxicos, particularmente em peixes, onde vários parâmetros comportamentais ecologicamente relevantes são fáceis de observar e de quantificar (Scott e Sloman, 2004). Alterações ao nível comportamental podem ter consequências prejudiciais directas sobre o organismo e na população (e.g. alterações na capacidade predatória, na fuga a predadores, ou no comportamento reprodutivo).

Alguns estudos desenvolvidos com espécies aquáticas têm mostrado que a inibição da ChE está associada a várias alterações comportamentais (Ballesteros *et al*., 2009; Vieira *et al*., 2009; Castro *et al*., 2009; Xuereb *et al*., 2009). Nos peixes, a inibição da AChE demonstrou estar associada a alterações da actividade locomotora, taxa de alimentação e crescimento (Castro *et al*., 2004; Sismeiro-Vivas *et al*., 2007; Ballesteros *et al*., 2009; Gravato e Guilhermino, 2009; Vieira *et al*., 2009).

3. Utilização de peixes em estudos ecotoxicológicos

A selecção dos organismos-teste para o desenvolvimento de ensaios ecotoxicológicos é feita de acordo com a disponibilidade do organismo (cultivados em laboratório ou disponibilidade no ambiente), distribuição geográfica (ensaio padronizado pode ser utilizado em diversas regiões de um país), relevância ecológica, duração do ciclo de vida, tolerância ao manuseio em laboratório, tolerância a factores abióticos como salinidade e pH, existência de metodologia padronizada e sensibilidade reconhecida (Peakall, 1992).

Os peixes são organismos sentinelas úteis, que permitem a detecção de efeitos de contaminantes ambientais e, como sistemas-modelo eficientes e rentáveis, têm sido seleccionados para estudos de toxicologia e avaliação de riscos durante décadas (Scott e Sloman, 2004; Lourenço *et al.*, 2010). Representam o grupo de vertebrados mais antigo e diversificado, possuindo a maior percentagem de espécies deste sub-filo, e ocupam um extraordinário leque de *habitats* (oceanos, mares, rios, lagos e fontes hidrotermais) (Bolis *et al.*, 2001). Devido aos diferentes tipos de *habitats* que ocupam, os peixes têm desenvolvido diferentes estratégias de sobrevivência, tornando-os particularmente úteis em estudos de ecotoxicologia, permitindo avaliar os efeitos de uma vasta gama de contaminantes sob um largo espectro de condições de exposição.

Os peixes são um excelente modelo para estes estudos, uma vez que o seu comportamento pode fornecer um índice de sensibilidade, para verificar o seu estado geral de saúde e stress (Ballesteros *et al.*, 2009). A exposição prolongada e intensa aos contaminantes pode induzir uma sequência de alterações comportamentais, funcionais e fisiológicas que prejudicam as funções vitais, como a capacidade dos peixes para a alimentação, evitar a predação ou reprodução. Pacheco *et al*. (1999) referem, que a adaptabilidade ao ambiente assenta numa complexa rede de respostas, como as alterações hormonais, fisiológicas ou metabólicas, que podem constituir excelentes indicadores da interacção dos organismos com o(s) agente(s) químico(s) que, directa ou indirectamente, afectam a qualidade ambiental. Os peixes desempenham um papel ecológico importante nas cadeias alimentares aquáticas, devido à sua função como transportadores de energia dos níveis tróficos inferiores para os superiores (Van der Oost *et al.*, 2003). A variedade de espécies de peixes, assim como dos seus habitats, são algumas das vantagens deste modelo biológico. Em estudos de monitorização ambiental, e de acordo com Zhou *et al.* (2008), para ser considerado um bom bioindicador, uma espécie deve: 1) poder acumular grande quantidade de poluentes sem morrer; 2) ser representativo de um determinado local; 3) apresentar uma elevada abundância e distribuição, permitindo repetição de amostragens; 4) apresentar uma longevidade que permita comparação entre diferentes idades; 5) ser de fácil amostragem e manutenção laboratorial; 6) apresentar posição importante na cadeia alimentar e 7) apresentar uma boa relação dose-efeito. Dado ser extremamente difícil encontrar um bioindicador que apresente todas estas características, a selecção do bioindicador deve ter em conta os objectivos específicos da biomonitorização (Zhou *et al.*, 2008).

Lepomis gibbosus (Perca-sol)

Lepomis gibbosus (perca-sol) é uma espécie invasora na Europa e tem-se tornado numa espécie planctívora/omnívora com maior incidência em lagos e reservatórios do Mediterrâneo, substituindo a comunidade indígena de ciprinídeos (Godinho e Ferreira, 1998; Garcia-Berthou e Moreno-Amich, 2000; Fox e Crivelli, 2001; Blanco *et al*., 2003; Castro e Gonçalves, 2007; Ozcan, 2007). Em termos de avaliação ecotoxicológica, esta espécie cumpre uma série de critérios exigidos para um organismo teste adequado: i) é fácil de identificar no campo, ii) é fácil de capturar e é abundante, iii) não está espécie ameaçada, iv) tem requisitos de manutenção laboratorial simples. A perca-sol apareceu nos nossos ecossistemas em 1977 (Godinho *et al.*, 1997) e é uma espécie bem-sucedida (Castro e Gonçalves, 2007), provavelmente devido à sua ampla tolerância às condições ambientais. Num cenário de monitorização ambiental, a comparação entre peixes desta espécie provenientes de diferentes *habitats* (poluídos e não poluídos) é possível. Além disso, antevemos o seu uso potencial como um organismo modelo, tanto em abordagens de monitorização como de

laboratório, aproveitando a sua capacidade de invasão - o que permite comparações entre países de vários continentes (em zonas temperadas, principalmente na Europa e América do Norte). Além de evitar o sacrifício de espécies nativas para estudos científicos, ela também apresenta vantagens relativamente aos peixes modelo comum, que são principalmente tropicais (*Poecilia reticulata*, *Oryzias latipes*, *Brachydanio rerio*, *Oreochromis* spp.), logo pouco representativos da zona temperada.

4. Objectivos e Estrutura da Dissertação

A presente dissertação pretendeu gerar dados ecotoxicológicos de alguns compostos anticolinesterásicos, numa espécie não alvo, para futuras avaliações de risco e/ou monitorização ambiental para três classes de compostos: detergentes (SDS), pesticidas (Clorfenvinfos) e fármacos (neostigmina e piridostigmina). Neste contexto, foi seleccionado o peixe *Lepomis gibbosus* (perca-sol) como organismo de estudo. Este apresenta-se como sendo um potencial candidato a espécie bio-indicadora, por se registar em abundâncias elevadas nos sistemas dulçaquícolas (sobretudo lênticos) portugueses, por ter requisitos simples de manutenção em laboratório, por não haver qualquer tipo de restrição à sua captura e sacrifício (trata-se de uma espécie não indígena), e por apresentar comportamentos facilmente mensuráveis.

Deste modo, os objectivos específicos da presente dissertação foram:

- Caracterizar a ChE presente em homogeneizados de cabeça e músculo dorsal em perca-sol (*Lepomis gibbosus*), através da utilização de diferentes substratos e inibidores selectivos; e avaliar os efeitos do detergente aniónico SDS (dodecilsulfato de sódio) e do insecticida organofosforado (clorfenvinfos) na AChE de *L. gibbosus* após exposição *in vitro* e *in vivo*.
- Avaliar o efeito de dois fármacos neurotóxicos na actividade da AChE (cabeça e músculo dorsal) e no comportamento de *L. gibbosus*.

De modo a facilitar a leitura, e de acordo com os objectivos anteriormente definidos, a presente dissertação encontra-se organizada em quatro capítulos. O primeiro capítulo corresponde à Introdução Geral onde é feita uma revisão da literatura relevante para o enquadramento do trabalho. Os Capítulos II e III apresentam-se escritos sobre a forma de artigos científicos e pretendem descrever o trabalho desenvolvido de modo a atingir os objectivos específicos previamente definidos. Por fim, o Capítulo IV traça as considerações finais relativas aos trabalhos apresentados nos dois capítulos anteriores, proporcionando o remate da presente dissertação.

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Capítulo II

Rodrigues S.R., Caldeira C., Castro B.B., Gonçalves F., Nunes B., Antunes S.C. (2011)

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Cholinesterase (ChE) inhibition in pumpkinseed (*Lepomis gibbosus*) as environmental biomarker: ChE characterization and potential neurotoxic effects of xenobiotics

Abstract

Inhibition of cholinesterases (ChEs) has been widely used as an environmental biomarker of exposure to organophosphates (OP) and carbamate (CB) pesticides. More recently, this biomarker has been suggested as a putative biomarker for exposure to detergents. The use of cholinesterase inhibition as effect criterion in Ecotoxicology requires the previous characterization of the specific enzymatic forms that may be present in different tissues or organs. Different ChEs isoforms may be present in the same tissue and may exhibit distinct sensitivities towards environmental contaminants. This work intended to characterize the soluble ChEs present in pumpkinseed sunfish (*Lepomis gibbosus*) total head and dorsal muscle homogenates, through the use of different substrates and selective inhibitors of cholinesterasic activity. Also, the *in vitro* effects of sodium dodecylsulphate (SDS – anionic detergent) and chlorfenvinphos (organophosphate pesticide) on the enzymatic activity of the mentioned species were investigated. In general terms, the predominant cholinesterasic form present in both tissues was acetylcholinesterase. Chlorfenvinphos was responsible for inhibitory effects on AChE activity, while SDS did not cause any significant effect. These results suggest that in environmental monitoring programs, *L. gibbosus* head and dorsal muscle AChE can be an adequate diagnostic tool for exposure to OP pesticides; this conclusion however is not applicable to detergent residues. We also discuss the usefulness of *L. gibbosus* as an alternative model system and valuable option for freshwater ecotoxicological monitoring programs.

Keywords: *Lepomis gibbosus*, cholinesterases characterization, environmental monitoring, enzyme inhibition, detergents, organophosphate pesticide, *in vivo* assays, in vitro assays, alternative model fish system, acetylcholinesterase (AChE) activity

Introduction

The increasing input of contaminants to aquatic ecosystems has generated the need to understand and evaluate the biological effects of pollutants on aquatic biota. In this sense, a large number of studies have used biomarkers as functional tools to evaluate the toxicity of such compounds for natural populations (Leticia and Gerardo, 2008). Biomarkers are interpreted, in ecotoxicological terms, as biological parameters that may suffer a significant alteration following

exposure to contaminants, as defined by Timbrell (Timbrell, 1998). For many years, ChEs have appeared to have potential as biomarkers for the monitoring of environment contamination by several compounds such as organophosphate (OP) and carbamate (CB) pesticides, heavy metals, mine effluents, pulp waste and detergents (Mora *et al.,* 1999; Castro *et al.,* 2004; Rodríguez-Fuentes and Gold-Bouchout, 2004; Li, 2008; Vieira *et al.,* 2009). During the 1990's there was a resurgence of interest concerning the use of ChEs as a biomarker, as evidence accumulated that ChEs activity in fish was inhibited at sites not obviously contaminated by organophosphate or carbamate pesticides. Although the chemical cause of such inhibition was still unidentified, ChEs was extensively used as an early warning bioassay to identify potentially contaminated sites which can be examined in more detail using the approaches of analytical chemistry (Jung *et al.,* 2007).

Cholinesterases (ChEs) belong to the family of enzymes designated as esterases, with the capability of hydrolyzing carboxylic esters. ChEs can be distinguished from other esterases since they exhibit preference for the hydrolysis of choline esters rather than other carboxylic esters and are inhibited by physostigmine (eserine) at concentrations in the range of 10^{-5}M (Garcia *et al.,* 2000; Nunes *et al.,* 2003). ChEs have been the most frequently used biomarkers to evaluate exposure and effects of several OP and CB pesticides (Rodríguez-Fuentes and Gold-Bouchout, 2004). Vertebrates have two types of ChEs: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase or pseudocholinesterase (BChE, EC 3.1.1.8), which differ in their substrate specificity (Mora *et al.,* 1999; Sturm *et al.,* 1999a; Leticia and Gerardo, 2008). Acetylcholinesterase (AChE), also known as true cholinesterase or specific cholinesterase, is important for the function of the nervous system. The main role of AChE is to catalyze the hydrolysis of acetylcholine into choline and acetic acid at cholinergic synaptic sites (Garcia *et al.,* 2000; Nunes *et al.,* 2003; Leticia and Gerardo, 2008).

A great diversity of organisms has been assayed for ChE activity including mammals, birds, fish, mollusks, crustaceans and insects (Garcia *et al.,* 2000; Varó *et al.,* 2002; Arufe *et al.,* 2007; Bervoets *et al.,* 2009). In fishes, AChE is predominant in brain and muscle tissues, whereas BChE is present mostly in the liver and plasma (Leticia and Gerardo, 2008; Sturm *et al.,* 1999a). Butyrylcholinesterase (BChE), the other cholinesterase present in the majority of vertebrates, is found mainly in the plasma, and has an unclear function (Jbilo *et al.,* 1994), but is thought to be involved in regulation of cell proliferation and the early stages of neuronal differentiation (Mack and Robitzki, 2000). Besides body fluids, BChE can exist in hematopoietic cells, liver, lung, heart, at cholinergic synapses, in the central nervous system, in tumours and in developing embryonic tissues (Mack and Robitzki, 2000). The inhibition of AChE provokes an accumulation of acetylcholine at the nerve synapses and disruption of the nerve function, frequently resulting in

death of the exposed organisms, as a consequence of overstimulation of the parasympathetic or autonomous nervous system (Nunes *et al.,* 2003).

Different cholinesterasic forms exhibit overlapping hydrolytic capabilities (Nunes *et al.,* 2003). In environmental monitoring, it is of fundamental importance to fully characterize the enzymatic form present in exposed organisms, and to know the normal range of activity in non-exposed individuals (Garcia *et al.,* 2000; Varó *et al.,* 2002). Several studies revealed that ChEs are polymorphic in most species (Garcia *et al.,* 2000; Varó *et al.,* 2002), and it is possible that different forms (AChE and BChE) show different sensitivity to anti-ChE agents. In practical terms, it is important to determine which type of ChE is most abundant in a candidate bioindicator species, as this will define which substrate (and concentration) is the most appropriate for monitoring purposes.

Lepomis gibbosus (pumpkinseed sunfish) is an invasive species in Europe and is presently becoming one of the most relevant planktivores/omnivores in many Mediterranean lakes and reservoirs, displacing the indigenous cyprinid community (Godinho *et al.,* 1998; Garcia-Berthou and Moreno-Amich, 2000; Fox and Crivelli, 2001; Ozcan, 2007; Blanco *et al.,* 2003). In terms of ecotoxicological assessment, it fulfills a series of criteria required for a suitable test organism: i) it is easy to identify in the field, ii) it is easy to capture and is abundant enough, iii) it is not under any type of legal protection, iv) it has simple laboratory rearing requirements. It is a well-successful species, probably because of its wide tolerance to environmental conditions, and we have recorded its presence in large numbers in polluted habitats, such as hypertrophic lakes (e.g. Castro and Gonçalves, 2007). In an environmental monitoring scenario, the comparison of fish health between polluted and non-polluted habitats is thus possible. Also, we envisage potential in its use as a model organism both in monitoring and laboratory approaches, taking advantage of its invasiveness – which makes it available in many countries across continents (warm temperate, mainly Europe and North America). Besides avoiding the sacrifice of native species for scientific studies, it also presents advantages relatively to common model fish, which are mostly tropical (*Poecilia reticulata*, *Oryzias latipes*, *Brachydanio rerio*, *Oreochromis* spp.).

The objectives of this study were to characterize the soluble ChE present in pumpkinseed sunfish (*Lepomis gibbosus*) head and dorsal muscle homogenates, through the use of different substrates and selective inhibitors, and to determine the normal range of activity in non-exposed specimens. Additionally, the effects of a widely employed anionic detergent (sodium dodecylsulphate, SDS) and organophosphorous insecticide (chlorfenvinphos) were investigated, both under *in vivo* and *in vitro* conditions. Comparisons between *in vivo* an *in vitro* assays were established in order to evaluate the implications of AChE use in the ecotoxicity assessment of contaminants such as detergents and OP insecticides. This and future studies will also allow to

evaluate *L. gibbosus* as a valuable alternative for freshwater ecotoxicological monitoring programs. The use of fish biomarkers as indices of pollution effects are of increasing importance and can permit early detection of environmental problems.

Materials and methods

Chemicals and test organisms

Acetylthiocholine, butyrylthiocholine, propionylthiocholine, 5,5'-dithio-bis (γ-nitrobenzoic acid) (DTNB), eserine hemisulfate salt, tetraisopropylpyrophosphoramide (ISO-OMPA), 1,5-bis (4 –allyldimethyl ammoniumphenyl)-pentan-3-one dibromide (BW284C51), lyophilised acetylcholinesterase from *Electrophorus electricus*, and bovine γ-globulin were purchased from SIGMA (Germany). Bradford reagent was purchased from BIO-RAD (UK). SDS (99% pure) and ethanol (99% pure) were purchased from MERCK (Germany). Test solutions of chlorfenvinphos [2-chloro-1-(2,4-dichlorophenyl) vinyl diethyl phosphate] were obtained by diluting its commercial formulation Quirlan® (24.64% pure, obtained from Sipcam–Quimiagro®, Portugal) in aged tap water.

Fishes were captured in Lake Vela, located in the Portuguese littoral-centre, which is a temperate shallow lake exhibiting a eutrophic status (see Castro and Gonçalves, 2007). Fishes were captured using a seine net (variable mesh size, 1-2 cm), which was deployed in a semi-circle and then pulled to the shore. After capture, fishes were kept under laboratory-controlled conditions (aged tap water, temperature 20±1°C, photoperiod 16 h light: 8 h dark and continuous aeration) for three weeks before experiments. Fish were fed daily with commercially available fish food (Sera Vipan®).

Enzymatic analysis

Fishes (between 1-2 g and 4-5 cm) were sacrificed by decapitation, and the head and dorsal muscle were analyzed individually. Samples were homogenized in ice-cold phosphate buffer (0.1 M, pH = 7.2). Homogenized tissues were centrifuged at 3300 × g for 3 min and supernatants were used for enzymatic determinations. Protein concentration in the samples was determined according to Bradford, 1976, adapted to microplate, in order to express enzymatic activities as a function of the protein content of the analysed samples. Prior to enzymatic assays, samples were normalized to an approximate protein content of 0.4 mg mL^{-1} for head and 1.0 mg mL^{-1} for muscle, based on previous optimization, see also (Castro *et al.*, 2004; Nunes *et al.*, 2005). Cholinesterase activity in head and dorsal muscle was determined spectrophotometrically in a Labsystem Multiskan EX microplate reader by the method of Ellman *et al.* 1961, adapted to microplate (as described by Guilhermino *et al.* 1996).

Characterization of cholinesterases

Cholinesterase characterization was carried out using the following enzyme-specific substrates: acetylthiocholine (specific for AChE), butyrylthiocholine (specific for BChE), and propionylthiocholine (specific for propionylcholinesterase). Substrate concentration varied from 0.005 to 5.12 mM. The following inhibitors were used: Eserine sulphate, BW284C51 and Iso-OMPA, which selectively inhibit total ChEs, AChEs, and BChEs, respectively. Inhibitor concentration was 6.25–200 µM for Eserine and BW284C51, and 0.25–8 mM for Iso-OMPA. Stock solutions of Eserine and BW284C51 were prepared in aged tap water, and Iso-OMPA stock solution was dissolved in ethanol. Each inhibitor solution (5 µL) was mixed with 495 µl of enzyme extract and then incubated at room temperature for 20 min (as described by Nunes *et al.* 2005). Aged tap water was used as a control, and an additional control was prepared with ethanol for the samples exposed to Iso-OMPA. Enzymatic activity was determined as above.

All assays followed a repeated measures (see Statistical Analysis) or treatment-by-subject design (Zar, 1996) to reduce the number of fish to sacrifice. The same three experimental subjects were used as replicate units for all levels (i.e. concentrations) of substrates and inhibitors, following a two-factor (concentration and subject) unreplicated design. To account for sample heterogeneity, each of these replicate units consisted of a pooled sample from three different individuals (muscle or head). In short, the sacrifice of only nine fish (3 x 3) was sufficient for the cholinesterase characterization assays (substrates and inhibitors) and *in vitro* experiments (see below). Each replicate measure was obtained as the mean value of four enzymatic determinations.

In vitro exposure to toxicants

We also studied the effect of SDS and Chlorfenvinphos exposure on AChE activity in the head and dorsal muscle. *In vitro* incubations were performed in 1.5 mL microtubes, by adding 5 µL of each test solution (dissolved in aged tap water) to a volume of 495 µL of supernatant (sample homogenate). Incubation was performed with gentle shaking at room temperature (20±1°C), during 30 min, using eight different concentrations of SDS (6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg L^{-1}) and Chlorfenvinphos (0.375, 0.75, 1.5, 3.0 and 6.0 µM). All concentrations were tested in triplicate and a control without toxicant (also in triplicate) was included. Enzymatic activity was determined according to the above-mentioned protocol.

We used the same group of pooled samples (as replicate units) used in the characterization assays (see above) in the *in vitro* exposures to toxicants (SDS and chlorfenvinphos), thus avoiding the additional sacrifice of animals. Correspondingly, we employed the same type of treatment-by-subject experimental design (see above).

In vivo exposure to toxicants

In vivo assays were performed through exposure of fish to sub-lethal concentrations of SDS and Chlorfenvinphos, for a period of 96 h. Ranges of concentrations of SDS and Chlorfenvinphos used in this study were chosen according to previous studies with *Gambusia holbrooki* (Nunes *et al.*, 2005; Sismeiro-Vivas *et al.*, 2007). Concentrations of SDS were: 2.8, 3.9, 5.5, 7.7, 10.8 and 15.1 mg L^{-1}; concentrations of Chlorfenvinphos were: 0.14, 0.20, 0.27, 0.38, 0.54 and 0.75 mg L^{-1}. Each assay had an independent control (non-exposed fishes). Fishes were individually exposed in 300 mL of test solution (prepared with aged tap water) in a total of 5 replicates per treatment. Abiotic conditions were controlled during the exposure period (photoperiod 16 h light: 8 h dark, temperature of 20±1°C, continuous aeration). No food was supplied during exposure. Exposure apparatus was composed of plastic containers, which had been thoroughly rinsed with aged tap water. Medium was renewed after 48 h from the start of exposure. Parameters such as mortality, pH, temperature and dissolved oxygen were monitored during exposure, for test validation purposes. After exposure, fishes were processed as above and AChE activity was quantified in head and muscle samples of each individual fish (see Enzymatic Analysis).

Data analysis

Data concerning the *in vitro* exposures to specific inhibitors and toxicants were analyzed with a mixed-model ANOVA (type III), using xenobiotic concentration and pooled sample (replicate unit or subject) as fixed and random factors, respectively. Data were log-transformed to comply with normality and homoscedasticity assumptions. In the presence of a significant effect of inhibitor or toxicant concentration, we used a Dunnett's test to assess which concentrations were significantly different from the control. In the case of Iso-OMPA, the control with ethanol was used as reference. Data from *in vivo* exposures to SDS and chlorfenvinphos were tested with one-way ANOVA, followed by the Dunnett's multicomparison test to discriminate significant differences between toxicant concentrations and the control. The significance level for all analyses was 0.05.

Results and Discussion

Cholinesterase characterization - head tissues

Incubation of both head and muscle samples from *L. gibbosus* with eserine showed a consistent and significant inhibition of the cholinesterasic activity (Table 1, Figure 2). Since eserine is a generic inhibitor of cholinesterases, it is possible to conclude that the predominant form present in both tissues is a cholinesterase, rather a non-specific esterase. Furthermore, incubation with the chemical BW284C51, the specific inhibitor of acetylcholinesterases, resulted in an almost complete (and significant) inhibition of all enzymatic hydrolytic activity, even for the lowest tested

concentration. The preference of both samples for specific substrates showed an unequivocal inclination for acetylthiocholine. Nevertheless, the other two substrates (butyrilthiocholine and propionilthiocholine) were also hydrolyzed, albeit at much slower rates (Figure 1). The hydrolytic degradation of acetylthiocholine was significantly higher, but this degradative tendency was inhibited at the highest concentrations of acetylthiocholine. These findings suggest that acetylcholinesterase must be the predominant enzymatic form, since this saturation behavior was already reported for similar organisms (Nunes *et al.*, 2005).

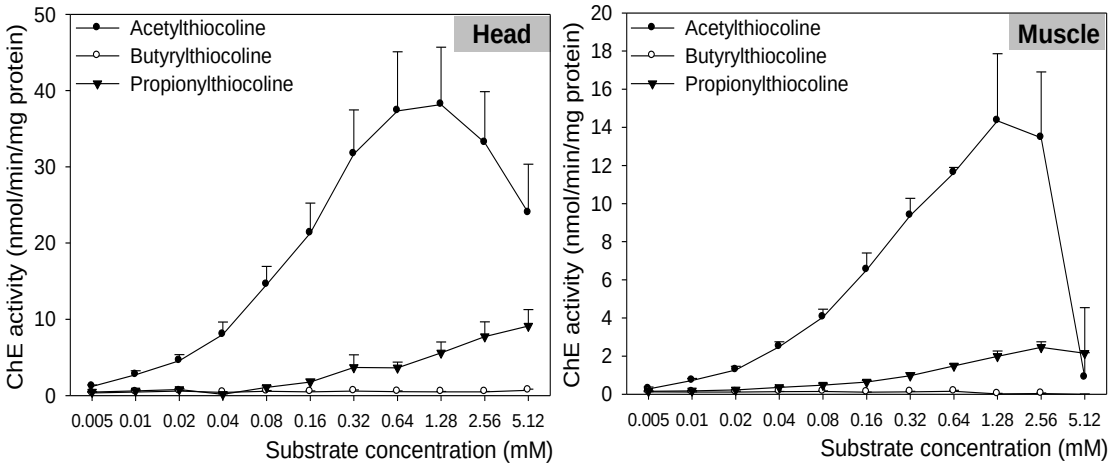

Figure 1 – Substrate preference of cholinesterases from total head and muscle homogenates of *L. gibbosus*.

Acetylcholinesterase is present in a large number of organisms, mainly in the nervous system. On these tissues homogenates, higher hydrolytic rates are always obtained with acetylthiocholine, and enzymatic activity is selectively inhibited by eserine and B284C51 (Figure 2). Results similar to the here-obtained data were previously described by a large number of researchers, in very distinct organisms. Varó *et al.* 2003 showed that the main cholinesterasic form in the brain tissue of the marine fish *Dicentrarchus labrax* was acetylcholinesterase. A similar conclusion was obtained by Rodríguez-Fuentes and Gold-Bouchot 2004, for the most predominant cholinesterase form present in brain the freshwater fish tilapia (*Oriochromus niloticus*). Arufe *et al.* 2007 showed that the predominant cholinesterasic form in gilthead seabream *Sparus aurata* larvae was acetylcholinesterase. Jung *et al.* 2007 conducted a study that pointed to the presence of acetylcholinesterase in the nervous tissue of the sole species *Limanda yokohamae*. The article by Sturm *et al.* 1999a registered the presence of acetylcholinesterase as the dominant form in brain tissue of several marine fish species, such as *Limanda limanda*, *Platichthys flesus* and *Serranus cabrilla*. Similar results were reported by Garcia *et al.* 2000 when studying the cholinesterase content of the freshwater fish species *Poecilia reticulata*. In agreement with these results, Nunes *et*

al. 2005 found that the most active cholinesterasic form found in nervous tissue of *Gambusia holbrooki* was acetylcholinesterase. Leticia and Gerardo 2008 characterized the cholinesterases of the fish species *Haemulon plumeri*, and found higher enzymatic activities, specifically measured as acetylcholinesterase, in brain tissue.

Acetylcholinesterasic predominance is not exclusive of vertebrates species. Key and Fulton 2002 showed that the grass shrimp *Palaemonetes pugio*, possessed acetylcholinesterase as the predominant cholinesterase form, evidenced by the higher preference for acetylthiocholine as substrate, and inhibition by eserine, BW284C51 (but not Iso-OMPA). Acetylcholinesterase was again found by Brown *et al.* 2004 in the gill homogenates of *Mytilus edulis*. The study performed by Frasco *et al.* 2006 referred that the main cholinesterasic form present in the eye tissues of the prawn *Palaemon serratus* was acetylcholinesterase. Similarly, the results obtained by Xuereb *et al.* 2007 showed that the crustacean *Gammarus pulex* most important cholinesterase was acetylcholinesterase, since it preferred acetylthiocholine as substrate and was inhibited by BW284c51.

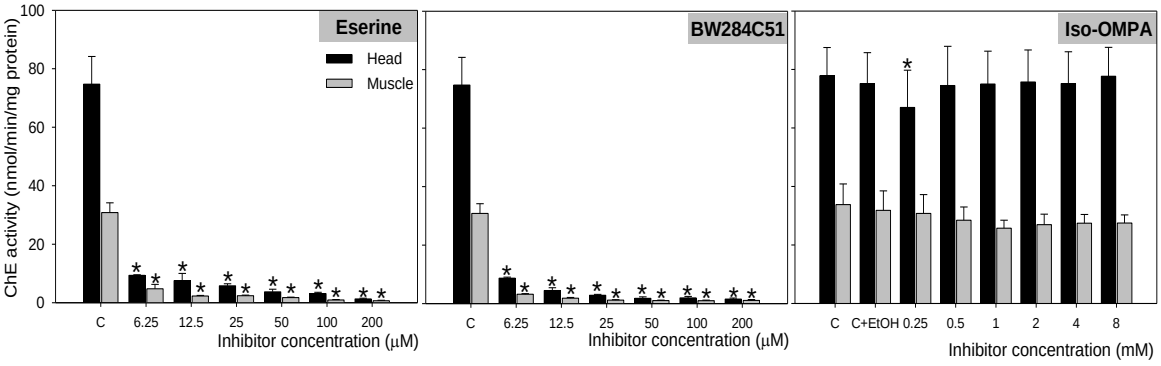

Figure 2 – Effects of specific inhibitors (eserine, BW284C51 and ISO-OMPA) on cholinesterase activity of total head and muscle homogenates of L. gibbosus. Values are the mean of three replicate assays and corresponding standard error bars. * Significant differences, $P \leq 0.05$.

Cholinesterase characterization - muscle tissue

Our results showed a consistent pattern, pointing to the involvement of acetylcholinesterase in muscle tissue of *L. gibbosus*: significant inhibition by eserine and BW284C51, and absence of effects after exposure to ISO-OMPA (Table 1, Figure 2). Furthermore, the preference profile is similar to the one observed for the head tissue homogenates, with stronger activities reported when using acetylthiocholine as substrate. A similar inhibitory tendency was also observed, but only for excess of substrate (Figure 1). However, muscle tissue is somewhat more difficult to systematize, since different cholinesterasic forms have already been described. Solé *et al.* 2008 evaluated the esterasic activity of muscle of the marine fish *Lipophrys pholis*, and concluded that atypical cholinesterasic forms were present in muscle tissue. Sturm *et al.* 1999b found a cholinesterasic

form with intermediate substrate preference in muscle tissue of *Gasterosteus aculeatus*, since it could degrade both acetylthiocholine and butyrilthiocholine.

Table 1 – RM (mixed-model) ANOVA summary table for the *in vitro* assays with specific ChE inhibitors and selected toxicants, in the head and muscle homogenates of *Lepomis gibbosus*. For each substance, the degrees of freedom (d.f.) and the variance between groups (MS) and within subjects ($MS_{subjects}$) are shown, as well as the *F* statistics (with associated *P* value) for the main factor (see text for additional explanation). NS stands for non significant.

	Inhibitors	d.f.	MS	$MS_{subjects}$	*F*	*P*
Head	Eserine	6, 11	0.70	0.062	259	**<0.001**
	BW284C51	6, 12	0.82	0.060	217	**<0.001**
	ISO-OMPA	7, 14	0.002	0.103	1.89	NS
Muscle	Eserine	6, 12	0.55	0.034	122	**<0.001**
	BW284C51	6, 12	0.57	0.002	106	**<0.001**
	ISO-OMPA	7, 14	0.003	0.109	1.93	NS
	Toxicants – in vitro					
Head	SDS	8, 16	<0.001	0.131	0.02	NS
	Chlorfenvinphos	5, 9	0.69	0.221	63.5	**<0.001**
Muscle	SDS	8, 16	<0.001	0.579	0.62	NS
	Chlorfenvinphos	5, 10	0.70	0.247	46.4	**<0.001**

Inhibitory effects caused by chlorfenvinphos

Cholinesterase inhibition of both head and muscle tissues after *in vitro* (Figure 3) and *in vivo* (Figure 4) exposures to chlorfenvinphos showed that this organophosphate pesticide was capable of exerting significant effects, even for the second lowest tested concentration. The experimental design allowed us to observe a NOEC value of 0.375 mg L^{-1}, for both tissues (Figure 3 and 4). One of the most interesting conclusions drawn from the here-obtained results is the similar responsiveness and sensitivity of head and muscle homogenates to this particular compound. Being an organophosphate pesticide, its function is directly linked to its inhibitory activity of acetylcholinesterase. AChE inhibition is the classic example of a specific biomarker that may be used in environmental assessment of a particular class of compounds, the anticholinesterasic pesticides (esters of the phosphoric acid and or the carbamic acid, organophosphate and carbamate pesticides, respectively).

The number of research articles in the field of Ecotoxicology that involve the use of AChE inhibition as effect criteria is vast. Aquatic organisms are extremely sensitive to the effects of anticholinesterasic pesticides, and serve as suitable sentinels for their presence in the environment. Sancho *et al.* 2000 observed a high responsiveness of ocular AChE activity in the eyes of European eel (*Anguilla anguilla*) after exposure to carbamate thiobencarb. The occurrence of cholinesterasic inhibition was showed by Ferrari *et al.* 2007, after studying the effects of the organophosphate azinphos methyl (AzMe) and the carbamate carbaryl, on juveniles of rainbow trout. Acetylcholinesterase activity was also inhibited in the hybrid catfish species (*Clarias macrocephalus* x *Clarias gariepinus*), following exposure to chlorpyrifos and carbaryl, as showed by Somnuek *et al.* 2009. Varó *et al.* 2002 showed a strong inhibitory effect after exposure of the crustacean species *Artemia salina* and *A. parthenogenetica* to the pesticides chlorpyrifos and dichlorvos. Brown *et al.* 2004 observed that the main cholinesterasic form present in *Mytilus edulis* gill homogenates was responsive to the pesticide azamethiphos. The acetylcholinesterase forms present in eye tissues of *Palaemon serratus* exhibited extreme sensitivity towards exposure to organophosphate and carbamate pesticides, as shown by Frasco *et al.* 2006. Iprobenfos is a pesticide that could also affect the acetylcholinesterase activity of aquatic organisms, as shown by Jung *et al.* 2007, when assessing the anticholinesterasic effects of this compound on *Limanda yokohamae*. According to these results, effects of chlorfenvinphos on the AChE of *L. gibbosus* are coherent with the established pattern of inhibition. This conclusion is of significant importance, considering the use of this particular species in environmental monitoring for the presence and effects of anticholinesterasic compounds in freshwater systems.

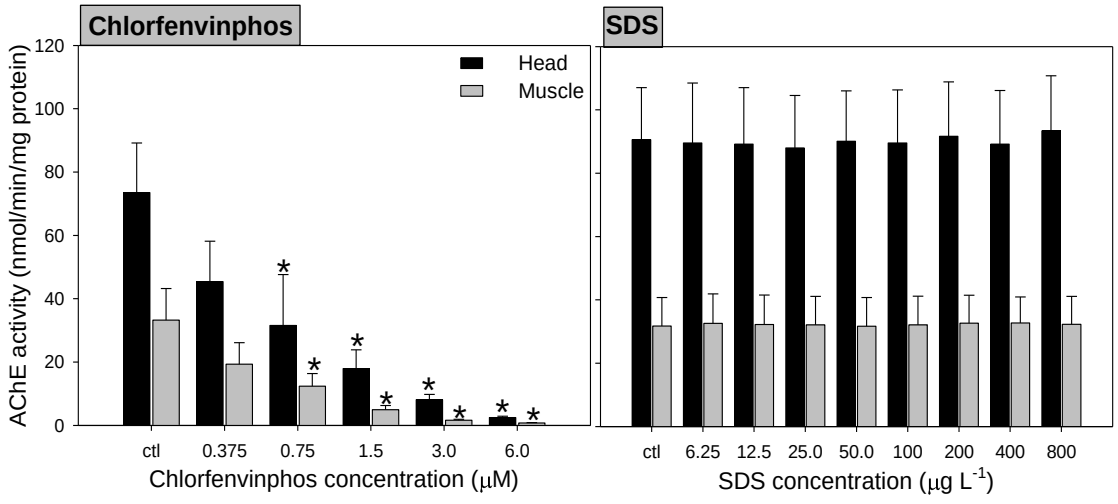

Figure 3 - *In vitro* effects of Chlorfenvinphos and SDS on acetylcholinesterase activity of total head and muscle homogenates from *L. gibbosus*. Values are the mean of three replicate assays and corresponding standard error bars. * Significant differences, $P \leq 0.05$.

Absence of effects caused by sodium dodecylsulphate

Our data showed an absolute lack of responsiveness of AChE after exposure to sodium dodecylsulphate. Both *in vitro* and *in vivo* exposure resulted in the absence of inhibitory effects of this particular detergent on the selected biomarker (Table 1 and 2, Figure 3 and 4). The here-obtained results are somewhat surprising considering previous work that showed an apparent inhibitory effect caused by detersive compounds on this enzyme. Inhibition of cholinesterases was reported for a significant number of non-organophosphate pesticides; compounds such as anionic detergents have been referred to significantly inhibit AChE activity *in vitro* in sensitive species, such as *Poecilia reticulata* (Garcia *et al.* 2000) and *Daphnia magna* (Guilhermino *et al.* 2000), both following *in vitro* and *in vivo* exposures. However, the work described by Nunes *et al.* 2005 evidenced that this inhibitory effect might be an artifact connected with the determination methodology. Detergents act by dissolving lipids, with the formation of tridimensional structures (micelles) that may entrap portions of membrane bound AChE molecules that are prevented to exert their degradatory activity. This work showed that micelles integrate AChE bound to lipid cellular membranes, preventing the enzyme to act on the chemicals used in the Ellman assay. Micelles formation can be prevented or reverted through the modification of the dieletric constant of the buffered media. By doing so, AChE can degrade the chemicals involved in the Ellman method (Ellman, 1961), reverting the alleged inhibitory effect caused by detergents. Furthermore, AChE inhibition may depend on the chemical nature of the tested detergents. Li, 2008 showed that inhibition of cholinesterase of the planarian species *Dugesia japonica* varied according the type of the tested detergent: inhibition was possible after exposure to hyamine 1622, pentadecafluorooctanoid acid, perfluooctane sulfonate, and 4 nonylphenol; enzymatic induction was caused by triton X-100.

Table 2 – One-way ANOVA summary table for the *in vivo* assays with selected toxicants, in the head and muscle samples of *Lepomis gibbosus*. For each substance, the degrees of freedom (d.f.), variance between groups (MS), and *F* statistics (with associated *P* value) are shown. NS stands for non significant.

Toxicants – in vivo		d.f.	MS	*F*	*P*
Head	SDS	6, 21	209	2.3	NS
	Chlorfenvinphos	6, 20	433	8.1	**<0.001**
Muscle	SDS	6, 19	767	1.6	NS
	Chlorfenvinphos	6, 17	511	2.6	NS

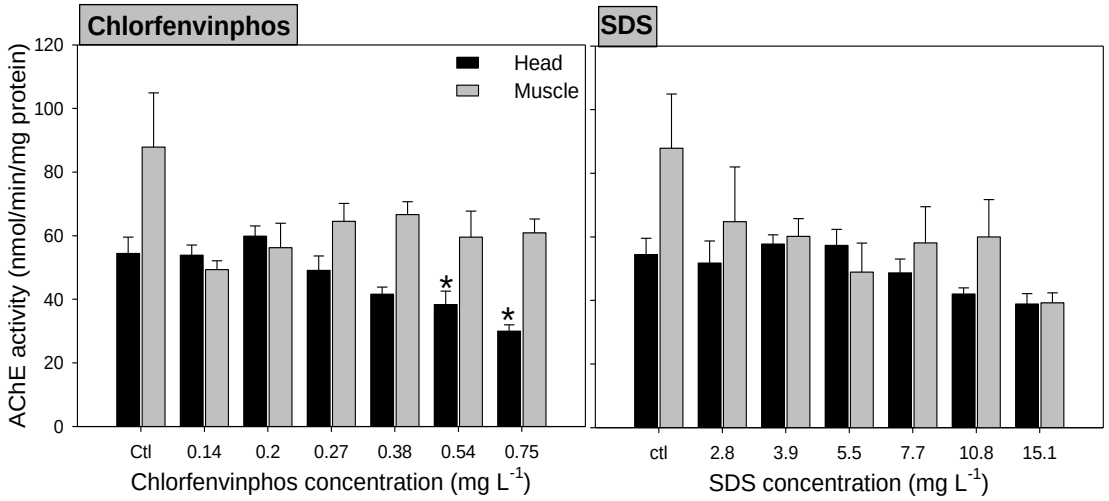

Figure 4 - *In vivo* effects of Chlorfenvinphos and SDS on acetylcholinesterasic activity of total head and muscle homogenates of *L. gibbosus*. Values are the mean of three replicate assays and corresponding standard error bars. * Significant differences, $P \leq 0.05$.

In terms of biological effects, other studies point to a lack of responsiveness, similar to the here observed, after exposure to similar compounds. Gonçalves *et al.* 2010 showed that exposure of *Gambusia holbrooki* to two distinct detergents (SDS and benzalkonium chloride) did not cause any significant alteration in the cholinesterasic activity of this fish species. Acetylcholinesterase hydrolytic activity is highly dependent on the electrostatic environment in which hydrolysis takes place, as shown by Malany *et al.* 1999. Substrates of these particular enzymatic forms move more easily into the catalytic gorge if they are cationic molecules (Massoulié *et al.*, 2008). It would not be surprising to observe that AChE activity may be theoretically modulated by a large number of ionic interactions with charged environmental contaminants, such as detergents. Alosteric interactions may also account for the potential inhibitory effects caused by charged compounds, since modifications in the protein tridimensional form, not in the active site, may justify the inhibitory activity reported for some compounds (Kitz *et al.*, 1970). Due to the diverse chemical (and electrical) nature of the detergent compounds that have been tested in terms of cholinesterasic inhibition, without effects, it is not possible to identify a straightforward pattern. In fact, the general rule of the cited articles is the absence of *in vivo* effects, in spite of occasional *in vitro* exposures that render inhibitory tendencies. It is not thus to exclude the possibility that the earlier reported inhibition of AChE by detergents may derive from an artifact of the protocol, rather than being a significant physiological impairment.

Usefulness of Lepomis gibbosus in environmental monitoring

Being an invasive species, with documented wide access to a large number of freshwater environments (Bhagat *et al.*, 2006; Vila-Gispert *et al.*, 2007), *Lepomis gibbosus* seems a promising

species for biomonitoring purposes. Our observations are in line with previously published data, obtained for fish species that share the same ecologic and geographical area, such as *Gambusia holbrooki* (Nunes *et al.*, 2005). Similarly, this study showed that SDS did not cause significant inhibitory effects following *in vivo* exposures, reinforcing the idea that fish cholinesterases may be refractory to detergent effects. Other freshwater or euryhaline species, such as eel (*Anguilla anguilla*) cholinesterases are also similar to the ones found in our study, as shown by Valbonesi *et al.* 2010, with a predominance of acetylcholinesterasic forms. Somewhat similar results were obtained for the estuarine fish species *Pomatoschistus microps*, a species that evidenced an atypical form of cholinesterase, able to degrade butyrilthiocholine whilst being inhibited by BW284C51 (Monteiro *et al.*, 2005). Exposure to an organophosphate compound yielded a significant inhibitory effect in our study; the study described by Ferrari *et al*. 2007 with the freshwater standard species *Oncorhynchus mykiss* showed a similar feature. It is thus possible to sustain that the responses observed with *L. gibbosus* in terms of responsiveness and physiological modifications were in complete agreement with previously described data, obtained for other freshwater species. This finding is of great importance since it allows us to propose the use of *L. gibbosus* as a valuable alternative for freshwater ecotoxicological monitoring programs, when assessing the presence and effects of anticholinesterasic compounds.

Conclusions

Our results showed that acetylcholinesterase is the main cholinesterasic form in total head homogenate of *L. gibbosus*. We envisage a large potential of this species as a model organism in ecotoxicology, and this study documents a first step towards the validation of this idea. Preference for acetylthiocholine as substrate, in parallel with the significant decrease in the hydrolytic activity with higher substrate concentrations, are distinctive features of an acetylcholinesterase, as already described for a large number of organisms. The inhibition profile by eserine and BW284C51 confirmed the presence of a specific form of acetylcholinesterase. The effects observed after *in vitro* and *in vivo* exposure to chlorfenvinphos and SDS pointed two basic trends: i) significant inhibition by the pesticide, not only in simple mechanistic terms (observed by the *in vitro* significant inhibitory effects), but also after uptake and disposition (made clear after *in vivo* exposures); ii) lack of effects consequent to the presence of SDS. This latter finding is of additional importance, since it shows that the use of AChE inhibition as effect criterion for the assessment of detergent exposure, using *L. gibbosus* as test organism, may not be adequate. When comparing our results with previously published data, it is clear that AChE inhibition as a consequence of detergent exposure is not likely to be an absolute rule. Our results support the view that the traditional role attributed to acetylcholinesterase inhibition for environmental assessment of

pesticide contamination is satisfactory. However, the alleged versatility of AChE inhibition as effect criteria after exposure to other non-specific classes of contaminants (including detergents) may be misleading and may underestimate the contamination potential of complex mixtures.

Acknowledgments

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Capítulo III

Anticholinesterase drugs effects on biomarkers and behavior of *Lepomis gibbosus* (pumpkinseed)

Abstract

The presence of pharmaceutical residues in the aquatic environment has recently received great attention, as potential adverse effects may arise from this presence. Inhibition of cholinesterases (ChE) has been widely used as an environmental biomarker of exposure to organophosphates (OP) and carbamate (CB) pesticides. However, other widespread anthropogenic contaminants (including pharmaceutical drugs) can exert toxic effects through ChE inhibition. Some studies with aquatic species have shown that inhibition of ChE is associated with behavioral changes. Bearing this in mind, this work aimed to study the effects on individual behavior and acetylcholinesterase (AChE) activity in selected tissues of *Lepomis gibbosus*, after exposure to the anticholinesterasic drugs, neostigmine and pyridostigmine. Animals were exposed to drugs for 96 h; 72 h after the start of the exposure, behavioral tests were conducted. The behavioral assessment consisted of determination the percentage of time in which organisms remained in the black part of the aquarium (% time in black), percentage of time in which organisms remained at the periphery of the aquarium (% time in the periphery) and percentage of time in which the organisms were in motion (% motion time), in two stages. Results revealed that neostigmine significantly decreased the activity of AChE in the head, but not in dorsal muscle. On the other hand, pyridostigmine significantly decreased the activity of AChE in both the head (in all concentrations) and dorsal muscle homogenates. The results of this study suggest that head AChE may be more sensitive than muscle AChE. In the behavioral assessment, there were no significant differences in the behavior of *Lepomis gibbosus* for all parameters, and for both drugs. The results suggested that the behavioral parameters analyzed in *L. gibbosus* cannot be regarded as a suitable marker to assess the effect of drugs such as neostigmine and pyridostigmine.

Keywords: neostigmine, pyridostigmine, acetylcholinesterase activity, behavior, *Lepomis gibbosus*

Introduction

An area of growing concern for both the public and the scientific community is the presence of pharmaceuticals in the environment, and the potential adverse effects these may have. The reason why pharmaceuticals may be interesting is that they are developed with the intention of causing a biological effect. Pharmaceuticals are a major class of chemical compounds, characterized by

continuous and indiscriminate use and biological activity (Daughton and Ternes, 1999; Halling-Sørensen *et al.*, 1998; Jones *et al.*, 2002; Miao *et al.*, 2002). They often have the same type of physico-chemical behavior - e.g. are lipophilic (in order to be able to pass biologic membranes) - and are persistent contaminants (Nunes *et al.*, 2005). Although pharmaceuticals are only present in surface waters at trace concentrations, typically in the range of ng – µg L^{-1}, some drugs can cause adverse effects at even lower concentrations, ≤ 1 ng L^{-1} (Fick *et al.*, 2010). In addition, combination (e.g. synergistic) effects may occur as a result of the simultaneous exposure to several different compounds in the environment (Cleuvers, 2003). Therefore, pharmaceutical drugs can be considered as environmental contaminants of particular concern. Moreover, high contamination values were previously described for several classes of therapeutic agents (Kümmerer, 2001), which can consequently lead to severe effects over non-target organisms.

Some of the chemicals that are used in human therapy exhibit pharmacological and toxicological properties that allow them to interfere with key processes, not exclusive to humans, such as neurotransmission. Neuromuscular transmission depends essentially on the release of acetylcholine and activation of the functionality of postsynaptic cellular receptors. This transmission can be affected, either by reducing or improving the availability of the neurotransmitter (namely, acetylcholine - ACh), or by dysfunction of the receptors (Ceremuga *et al.*, 2002). The compounds neostigmine and pyridostigmine are well known cholinesterase inhibitors that have been extensively used over the last five decades in the treatment of neuromuscular junction disorders, epitomized by the autoimmune disorder myasthenia gravis (autoimmune disease), and non-autoimmune congenital myasthenic syndromes, and have additionally been used as prophylactics for organophosphate poisoning (Gold and Schneider-Gold, 2008; Argov, 2009; Yu *et al.*, 2010). In certain diseases, such as myasthenia gravis, the production of antibodies against the acetylcholine receptor takes place, thus decreasing the number of ion channels activated by ACh and seriously compromising the transmission at the neuromuscular junction (Ceremuga *et al.*, 2002). In some cases, the cholinesterase inhibitors neostigmine and pyridostigmine are used to treat the disease, and consequently allow a longer presence of the neurotransmitter ACh in the synaptic cleft, making more likely its binding to the few remaining ACh receptors (Ceremuga *et al.*, 2002) and increasing the overall effect. These anti-cholinesterasic compounds are highly water-soluble (Yu *et al.*, 2010). ChEs appear to have potential as biomarkers for the monitoring of environment contamination by several compounds such as organophosphate (OP) and carbamate (CB) pesticides, heavy metals, mine effluents, pulp waste, pharmaceuticals and detergents (Mora *et al.*, 1999; Castro *et al.*, 2004; Rodríguez-Fuentes and Gold-Bouchout, 2004; Nunes *et al.*, 2006; Li, 2008; Vieira *et al.*, 2009; Gonçalves *et al.*, 2010). Acetylcholinesterase (AChE; EC 3.1.1.7), also known as true cholinesterase or specific

cholinesterase, is a key enzymatic form in the mechanism of neurotransmission, since it hydrolyses the neurotransmitter acetylcholine after its release at the nervous cleft of cholinergic synapses (Van der Oost *et al.,* 2003). The main role of AChE is to catalyze the hydrolysis of acetylcholine into choline and acetic acid at cholinergic synaptic sites (Garcia *et al.,* 2000; Nunes *et al.,* 2003; Leticia and Gerardo, 2008), thus ending the propagation of the nervous impulse. AChE inhibition leads to overstimulation of the central and peripheral nervous systems, resulting in deleterious neurotoxic effects in organisms, which can lead to death (Xuereb *et al.,* 2009).

Despite the high value of biomarkers as early warning tools, the significance of some environmental studies based on biomarkers has been questioned mainly due to the fact that alterations induced at sub-individual level do not necessary have negative effects at higher levels of biological organization (Vieira *et al.,* 2009). Without questioning the general accuracy of the argument, it is our opinion that the problem is the lack of knowledge on relationships between biomarkers and parameters considered ‘‘ecologically relevant” and, thus, that more research is needed on this matter to take full advantage of these powerful tools. It is important to establish direct linkages between biochemical and physiological impairments (e.g. enzymes) and behavioral alterations that may denote disturbances at the population level (Little *et al.,* 1990; Scott and Sloman, 2004; Vieira *et al.,* 2009). Relationships between AChE inhibition and impairment of physiological and behavioral processes have been studied mostly in vertebrate species (Castro *et al.,* 2004; Sismeiro-Vivas *et al.,* 2007). Fish are an excellent model in this regard, since many ecologically relevant fish behaviors are easily observed and quantified in a controlled setting. Several studies confirm that exposure to toxicants such as metals, polycyclic aromatic compounds, pharmaceuticals, and pesticides causes altered behavioral patterns in laboratory (Scott and Sloman, 2004; Correia *et al*., 2007; Sismeiro-Vivas *et al*., 2007; Castro *et al*., 2009; Vieira *et al*., 2009). In fish, AChE inhibition has been shown to modify swimming performance, food consumption and growth (Castro *et al*., 2004; Sismeiro-Vivas *et al*., 2007; Ballesteros *et al*., 2009; Xuereb *et al*., 2009). Behavior indeed conditions the individual’s ability to directly cope with its surrounding environment and ultimately to reproduce and survive (Engenheiro *et al*., 2005).

Rodrigues *et al.* (2011) showed that AChE is the main cholinesterasic form in total head and dorsal muscle homogenates of *L. gibbosus*. Bearing this in mind, this study intended to evaluate the effect of anticholinesterasic drugs neostigmine and pyridostigmine in AChE activity and behavior of *L. gibbosus*. The evaluation of behavioral changes was performed by allocating time spent by the fish according to motion type and in pre-defined regions of the aquarium. Measured behavioral endpoints are viewed as a measure of anxiety (see Maximino *et al*., 2010).

Materials and methods

Chemicals and test organisms

Acetylthiocholine, 5,5'-dithio-bis (γ-nitrobenzoic acid) (DTNB) and bovine γ-globulin were purchased from SIGMA ALDRICH (Germany). Bradford reagent was purchased from BIO-RAD laboratories (UK). Neostigmine bromide (CAS number 114-80-7) and pyridostigmine bromide (CAS number 101-26-8) were purchased from SIGMA-Aldrich (Madrid).

Lepomis gibbosus (pumpkinseed sunfish) is an invasive species in Europe and is presently becoming one of the most relevant planktivores/omnivores in many Mediterranean lakes and reservoirs, displacing the indigenous cyprinid community (Godinho and Ferreira, 1998; Garcia-Berthou and Moreno-Amich, 2000; Fox and Crivelli, 2001; Blanco *et al.*, 2003; Castro and Gonçalves, 2007; Ozcan, 2007). Rodrigues *et al*. (2011) refers a series of criteria required for a suitable test organism, and discusses the advantages of using pumpkinseed as a model organism in ecotoxicological studies.

Fishes were captured in Lake Vela, located in the Portuguese littoral-centre, which is a temperate shallow lake exhibiting a eutrophic status (more information, see Castro and Gonçalves, 2007). Fishes were captured using a seine net (variable mesh size, 1-2 cm), which was deployed in a semi-circle and then pulled to the shore. After capture, fishes were kept for a period of quarantine under laboratory-controlled conditions (aged tap water, temperature 20±1°C, photoperiod $16h^{L}$: $8h^{D}$ and continuous aeration) for three weeks before experiments. Fish were fed daily with commercially available fish food (Sera Vipan®).

Fish 96-h (acute) exposure

Fish (between 1-2 g and 4-5 cm) were exposed to a range of concentrations of neostigmine and pyridostigmine, for a period of 96 h. Ranges of concentrations used in this study were chosen based on previous studies (Richardson and Bowron, 1985; Sanderson and Thomsen, 2009; Fick *et al.*, 2010). The study by Fick *et al.* (2010) presented the predicted critical effect concentrations (CECs) for 500 pharmaceuticals expected to bioconcentrate from water to a steady state fish blood plasma concentration equal to the human therapeutic blood plasma level (concentration ratio = 1). Thus, CEC for neostigmine is 40.059 ng L^{-1} and for pyridostigmine is 5.4E+06 ng L^{-1}. Another study (Sanderson and Thomsen, 2009) presented a fish acute LC_{50} for pyridostigmine that was above 100 mg L^{-1}. Richardson and Bowron (1985) reported levels in the environment of pyridostigmine bromide of 0.22 µg L^{-1} in the river Lee, London, United Kingdom. According to this set of data, it was possible to define ecologically relevant exposure levels to which fishes were subjected. Concentrations chosen for neostigmine and pyridostigmine were: 10, 100, 1 000, 10 000 and 100 000 µg L^{-1} and 1, 10, 100, 1 000, 10 000 and 100 000 µg L^{-1}, respectively. Each assay had an

independent negative control (unexposed fishes). Test solutions of both neostigmine and pyridostigmine used for exposures were prepared by dilution of a stock solution in dechlorinated tap water, which was freshly prepared prior testing. Fishes were individually exposed in 500 mL of test solution, in a total of 5 replicates per treatment. Abiotic conditions were controlled during the exposure and were similar to quarantine period (photoperiod $16h^{L}$: $8h^{D}$, temperature of 20±1°C, continuous aeration). No food was supplied during exposure. Exposure apparatus was composed of plastic containers, which had been thoroughly rinsed with deionized water. Medium was renewed 48 h after the start of exposure. Behavioral tests were conducted 72 h after the start of exposure (see description below). Mortality, pH, temperature and dissolved oxygen were monitored during exposure for test validation purposes. After exposure (96 h), fishes were sacrificed and processed for the determination of AChE activity (see Enzymatic analysis).

Behavioral test

Behavioral trials were performed 72 h after the onset of exposure. Animals were transferred from plastic containers to the test chamber (modified aquarium) using fishnets. We used the apparatus (Figure 1) and protocol proposed by Maximino *et al.* (2010), with adjustments.

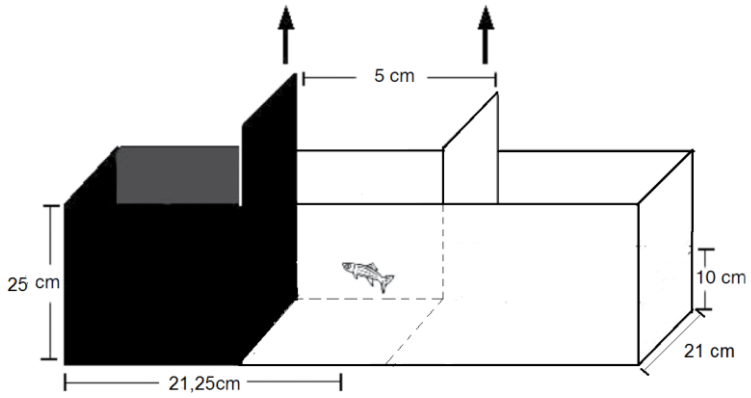

Figure 1 – Test apparatus used to perform behavioral trials (adapted from Maximino *et al.*, 2010).

The test apparatus (see Figure 1) consisted in an aquarium (42.5 cm length × 21 cm width × 25 cm height) divided equally into one-half black and one-half white, with a central area (without color). The central colorless area (5 cm × 21 cm × 10 cm) was separated from the black and white compartments with sliding doors, which were either black or white, to warrant uniform zone for each compartment. For the behavioral trials, the water column was kept at 10 cm, yielding a final volume of 9 L. Medium was composed of clean aged tap water. All exposed fishes were used in the behavioral test. Briefly, animals were individually placed in the colorless central compartment for a short acclimation period (approximately 20 s). After this period, the doors were gently removed to assess the preference of the fish (black versus white compartment). This procedure is based on

previous studies, which observed the natural preference of several fish species for the black compartment (scototaxis), where it feels more protected (Maximino *et al.*, 2010). The behavior of fish was continuously recorded in a fixed monitoring area (all compartments in the aquarium) with a high resolution JVC digital video camera (Model No: GR-b239E) mounted 40 cm above the aquarium test. The behavioral pattern was recorded in video sequence, and was digitalized in a personal computer. Behavioral responses, in particular the region of the aquarium, are easy to evaluate, without much need of an experimented observer.

Monitorization of behavior was undertaken in two stages. Stage 1 corresponded to the period of time between the removal of the sliding doors (shown in Figure 1), and the immobilization of fish (for more than 20 s) in a region of the aquarium. At this moment, the fish was stimulated by gentle prodding of its caudal fin, forcing it to move to another compartment. This began the observational stage 2, which continued until the fish immobilized itself (for more than 20 s). For each stage, the same parameters were evaluated: percentage of time in the black compartment (% time in black), percentage of time in the periphery of the aquarium (% time in periphery), and percentage of time in motion (% time in motion). These three measures were independent from each other and were all obtained from inspection of the timeline of video recordings, taking into account the total time of each stage. The periphery was considered the area from the wall of the aquarium up to 2 cm away from it. This region was defined based in preliminary studies, as unexposed fish often demonstrated a tendency towards the periphery. After the behavioral trial, fish were removed carefully from the test aquarium and transferred back into the test containers, to continue the exposure to the pharmaceutical compounds for additional 24 h (i,e, a total of 96 h).

Enzymatic analysis

Fishes (5 per treatment) were sacrificed by decapitation, and the head and dorsal muscle were isolated and individually analyzed. Head and muscle samples were homogenized in ice-cold phosphate buffer (0.1 M, pH = 7.2). Homogenized tissues were centrifuged at 3300 g for 3 min, and supernatants were used for enzymatic determinations. Protein concentration in the samples was determined according to Bradford (1976), adapted to microplate, in order to express enzymatic activities as a function of the protein content of the analyzed samples. Prior to enzymatic assays, samples were normalized to an approximate protein content of 0.4 mg mL^{-1} for head and 1.0 mg mL^{-1} for muscle, based on previous optimization (see also Castro *et al.*, 2004; Nunes *et al.*, 2005). Acetylcholinesterase activity in head and dorsal muscle was determined spectrophotometrically in a Labsystem Multiskan EX microplate reader by the method of Ellman *et al.* (1961), adapted to microplate (as described by Guilhermino *et al.*, 1996).

Data analysis

Head and muscle AChE activity data from exposures to neostigmine and pyridostigmine were statistically analyzed with one-way ANOVA, followed by the Dunnett's multicomparison test (if ANOVA was significant) to discriminate significant differences between toxicant concentrations and the control treatment.

Behavioral data (% time in black, % time in periphery, % time in motion) were arcsine-transformed prior to statistical analysis. Each variable was analyzed separately with one-way ANOVA, followed by the Dunnett's multicomparison test (if ANOVA was significant) to discriminate significant differences between toxicant concentrations and the control treatment, in relation to behavior.

All analyses used a significance level (α) of 0.05.

Results

Acetylcholinesterase activity

Exposure to neostigmine caused a significant decrease in the activity of AChE of total head homogenates in the highest tested concentration (100 000 μg L^{-1}; $F = 13.6$, d.f. = 5, 23; $p < 0.001$; Fig. 2). On the contrary, animals exposed to neostigmine did not present consistent alterations in the activity of AChE of muscle homogenates among treatments ($F = 1.81$, d.f. = 5, 23; $p = 0.151$; Fig. 2).

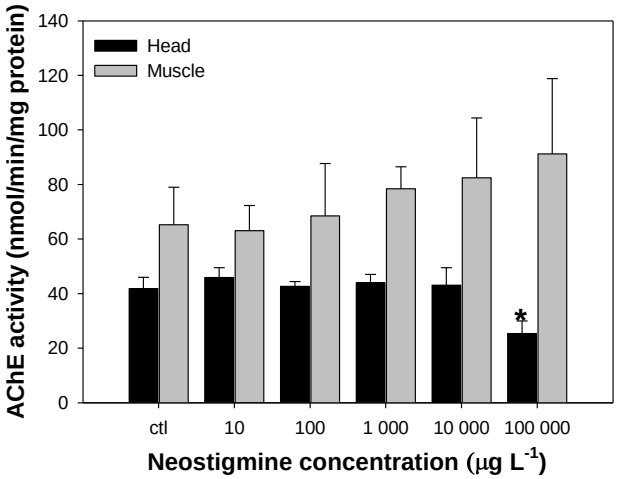

Figure 2 - Effects of neostigmine on the AChE activity from total head and muscle homogenates of *L. gibbosus.* Values are the mean of five replicate assays and corresponding standard error bars. * stands for statistically significant differences relatively to control (ctl), $p \leq 0.05$.

Pyridostigmine caused a significant decrease ($F = 6.68$, d.f. = 6, 28; $p < 0.001$; Fig. 3) in the activity of AChE of total head homogenates in all concentrations tested (> 1 μg L^{-1}). The activity of AChE in muscle was also significantly decreased ($F = 5.09$, d.f. = 6, 22; $p = 0.002$; Fig. 3), but only for the highest concentration (100 000 μg L^{-1}).

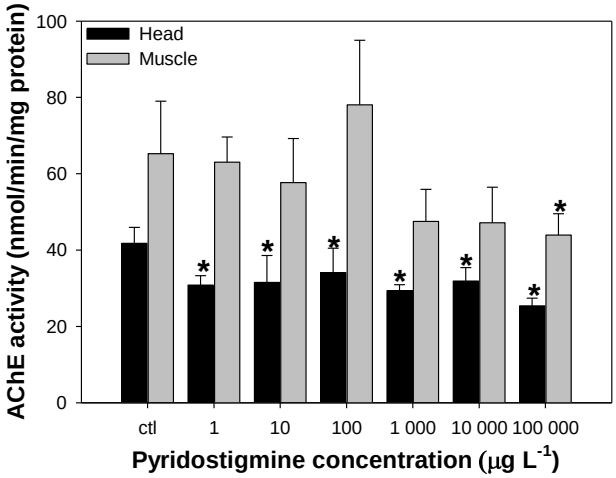

Figure 3 - Effects of pyridostigmine on the AChE activity from total head and muscle homogenates of *L. gibbosus.* Values are the mean of five replicate assays and corresponding standard error bars. * stands for statistically significant differences relatively to the control, $p \leq 0.05$.

Behavioral test

No statistical differences were found among experimental treatments for any of the behavioral endpoints, neither for neostigmine nor pyridostigmine (Table 1).

Table 1 - One-way ANOVA summary table for the behavioral trials with selected toxicants (neostigmine and pyridostigmine) in *L. gibbosus*. For each substance, the degrees of freedom (d.f.), F statistics and associated P value are shown. The behavioral trial was divided in two stages, which were analysed separately.

Toxicants			d.f.	F	P
Neostigmine	Stage 1	% time in black	5, 23	1.060	0.408
		% time in periphery	5, 23	0.829	0.542
		% time in motion	5, 23	1.215	0.334
	Stage 2	% time in black	5, 23	2.501	0.060
		% time in periphery	5, 23	0.661	0.657
		% time in motion	5, 23	0.322	0.895
Pyridostigmine	Stage 1	% time in black	5, 22	1.160	0.360
		% time in periphery	5, 22	1.667	0.184
		% time in motion	5, 22	0.370	0.864
	Stage 2	% time in black	5, 22	0.376	0.859
		% time in periphery	5, 22	1.353	0.280
		% time in motion	5, 22	0.775	0.578

There was a large variability in the responses of fish within the same concentration of neostigmine and pyridostigmine, so that the variability resulted in large standard errors and consequent lack of statistical differences compared to controls (Figures 4 and 5; Table 1).

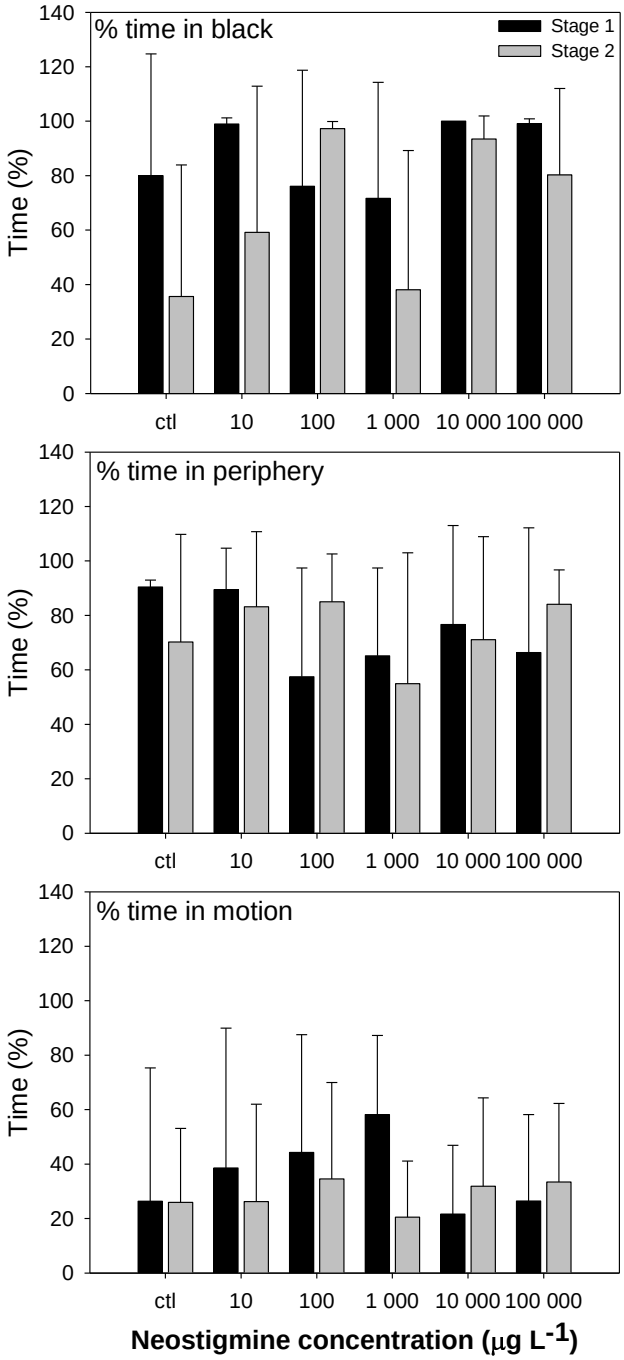

Figure 4 - Effects of neostigmine on the behavior of *L. gibbosus*. Values are the mean of five replicate assays and corresponding standard error bars.

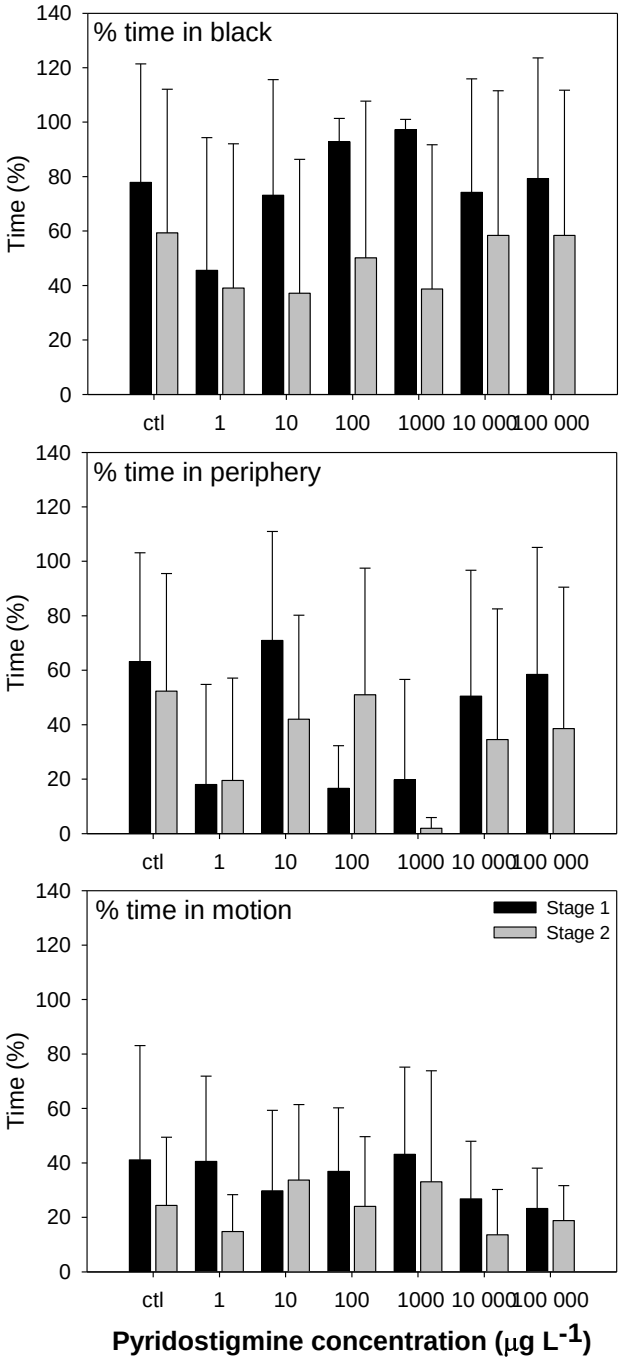

Figure 5 - Effects of pyridostigmine on the behavior of *L. gibbosus*. Values are the mean of five replicate assays and corresponding standard error bars.

Discussion

Inhibition of AChE activity after exposure to neostigmine and pyridostigmine

A review of the available scientific literature revealed little information on the toxicity of neostigmine and pyridostigmine in aquatic vertebrates, such as fish. Some studies have been

carried out to assess the effects after exposure to these compounds (neostigmine and pyridostigmine) in mammals, including rats (Kempen *et al.*, 1999; Joosen and Helden, 2007; Lamproglou *et al.*, 2009; Choi *et al*., 2010) and humans (Mirakhur *et al.*, 1982; Ceremuga *et al.*, 2002; Yu *et al.*, 2010; Zimerman *et al.*, 2010), which confirmed its anticholinesterasic properties. Our study is one of the few studies involving the effects of neostigmine and pyridostigmine on the activity of AChE in head and muscle of aquatic organisms. Fick *et al*., (2010) proposed that the CEC (predicted critical effect concentrations) concept could be applied to identify drugs with a potential for causing adverse environmental effects at measured environmental concentrations through mechanisms that are conserved between aquatic vertebrates and humans. The conceptual approach of Huggett *et al*. (2003), or the so-called fish plasma model was invented to estimate potential hazardous compounds and to prioritize experimental testing with fish. This model is based on a certain plasma concentration of a pharmaceutical that is required to affect the target (e.g. receptor, enzyme) in humans, and therefore approximately the same level would be required to affect another species sharing the same target (Huggett *et al*., 2003). Due to the conservative nature of physiological processes, many aquatic species at higher trophic levels, e.g. fish and amphibians, possess target molecules similar to human or veterinary drug targets (Fent *et al*., 2006). It is assumed that the risk for adverse effects in the environment increases with the degree of homology between human drug target and potential targets in lower vertebrates. Homology between human and fish receptors has already been shown by Christen et al. (2010), who evidenced > 45% homology between human and fish receptors targeted by specific pharmaceutical drugs (e.g. estrogen receptor, progesterone receptor, androgen receptor, thyroid hormone receptor, retinoic acid receptor RAR, adrenoreceptor, GABA receptors).

All drugs tested in this study were considered to have the same mode of action in humans and fish. In their study, Fick *et al*. (2010) presented a predicted critical effect concentration (CEC) for neostigmine of 40.1 ng L^{-1} and for pyridostigmine of 5.4E+06 ng L^{-1}. Thus, concentrations higher than those suggested by Fick et al. (2010) may cause toxic effects in exposed organisms. Our results are consistent with these data for neostigmine and pyridostigmine, as the concentrations tested in our study induced inhibitory effects on the activity of muscle or head AChE. For neostigmine, a NOEC value of 10 000 µg L^{-1} was observed in the head. For pyridostigmine, significant changes in AChE activity were observed in the head, for all tested concentrations (NOEC < 1 µg L^{-1}). However, a NOEC of 10 000 µg L^{-1} was observed in the muscle, for the same substance. With regard to pyridostigmine, note that Sanderson and Thomsen (2009) present a 96-h LC_{50} > 100 mg L^{-1} for fish. In our study, various concentrations of pyridostigmine were tested without recording mortality, with the highest concentration being 100 mg L^{-1}.

Cholinergic nervous function of *L. gibbosus* did not seem to be significantly altered following acute exposure to neostigmine, since AChE inhibition only occurred in the head and in the last concentration tested (100 000µg L^{-1}). It is possible, however, that neostigmine is scavenged by a prior barrier, before reaching the brain. Gonçalves *et al*. (2010) suggested the possibility of induction of serum cholinesterases in other animal species to clofibrate and diazepam. Salles *et al*. (2006) refer in their study that cholinesterase from piaussu (*Leporinus macrocephalus*) serum is three orders of magnitude more able of binding methyl-paraoxon than brain AChE, which suggests that organophosphate molecules in piaussu blood will be efficiently scavenged by serum cholinesterase before inhibiting brain AChE.

On the other hand, the study of Aquilonius *et al*. (1983) showed that the pharmacokinetic profiles of the two drugs were fundamentally similar with a terminal half-life in plasma of about 1-4 h and 0-9 h for pyridostigmine and neostigmine, respectively, in patients with myasthenia gravis. The oral bioavailability was higher for pyridostigmine (7-6%) than for neostigmine (2%), which in combination with the longer terminal half-life might offer some pharmacokinetic advantages in maintenance therapy. The study of McNamara *et al*. (2008) indicated that the half-life of neostigmine is short, ranging from 25 to 80 min, in patients with colonic pseudo-obstruction, after single injection. Neostigmine has short duration of action (30 min to 2 h) (Infarmed, 2007). Pyridostigmine has an duration of action 3-6 hours (Infarmed, 2006), therefore, larger than the neostigmine. The short-life of neostigmine could partly explain why no AChE inhibition was not found in low and medium concentrations, unlike for pyridostigmine.

Our results point to a low, almost null, interaction of neostigmine with the cholinesterasic activity in the central nervous system of *L. gibbosus.* The way that animals are exposed to test compounds can also be a determining factor in the toxic response obtained, and can be accounted for the differences of effects observed. The majority of results presented and obtained from the literature refer to mammals receiving the test compounds through injection or diet. This fact increases the difficulty in the extrapolation of comparisons to our experimental conditions because we used neostigmine directly dissolved in the test medium. In the aquatic environment, the permanent contact of fish with the water promotes the direct absorption of contaminants via respiratory surface (gills) and to a lesser degree, through the skin (Walker *et al*., 2001). Absorption can also occur through the digestive system via food. In our study, drugs were diluted into the surrounding environment. As such, absorption may have occurred directly from it or through the gills, which are documented as a first barrier to contaminants (Walker *et al.*, 2001).

Exposure to pyridostigmine caused a significant inhibition of head AChE activity in all concentrations tested, demonstrating that this response may not be dose dependent. However, further studies with lower concentrations should be conducted to confirm this assumption.

Pyridostigmine has a relatively short inhibition action on ChE compared to organophosphorus inhibitors (Song *et al*., 2002). A study by Lamproglou *et al*. (2009) tested the effect of pyridostigmine in rats, but did not observe acute cholinergic signs in pyridostigmine treated animals. Pyridostigmine is a polar chemical at physiological pH (containing a quaternary ammonium group) and thus would not be expected to readily distribute across the blood brain barrier (BBB) to act within the CNS and inhibit AChE (Song *et al*., 2002; Yu *et al*., 2010).

Different hypotheses have been presented in several studies to explain the effects of pyridostigmine in the central nervous system in rats. Song *et al.* (2004) suggested that stress induces alterations in blood brain barrier (BBB) permeability and it can allow penetration of pyridostigmine or one of its metabolites in the brain. However, no factor of stress was used in our study and we obtained inhibition of AChE. For other hand, pyridostigmine can gain access into the brain through fenestrated capillaries in different regions of the CNS, which are relatively devoid of BBB. However and considering this hypothesis, pyridostigmine could have behavioral effects even in non-stressed rats, which was not confirmed in the study of Lamproglou et al. (2009). In our study, there were no differences in behavior in fish exposed to pyridostigmine, compared with the control fish. The other hypothesis presented, could be an action of pyridostigmine on esterase activity in brain capillary endothelial cells resulting in alteration of BBB permeability for physiological substrates or xenobiotics resulting in neurodeterioration as suggested by different works, listed by Lamproglou *et al.* (2009).

As we can see by our results, the AChE of the head seemed to be more sensitive than that present in muscle tissue. Muscular AChE represents the largest pool of cholinesterases in the body, and is important for controlling the muscular function (Ballesteros *et al*., 2009). Results from the study of Pathiratne *et al.* (2010) assessed the overall effect of contamination on lake fish, demonstrating that muscle cholinesterases are more sensitive than brain cholinesterases. More than one form of ChE may be present in different tissues of the fish and these different forms have distinct sensitivities to anticholinesterasic agents (Sturm *et al*. 2000). Fish brain contains AChE, but no BChE, while muscle tissues may contain both AChE and BChE, depending on the species (Sturm *et al*., 1999; Sturm *et al*., 2000). Therefore, it is essential to perform the characterization of the ChE present in the tissue and species to be used in toxicological assays (Nunes *et al*., 2005). In mammals, gene inactivation studies of AChE have been complicated by the presence of a sister enzyme that can hydrolyze acetylcholine, BChE (Downes and Granato, 2004). BChE is transcribed from a distinct gene and it is structurally similar to AChE, although its function is not understood. It is expressed in many of the same tissues, albeit at much lower levels, and it likely compensates for AChE deficiencies. Although the predominant enzymatic form in muscle is AChE (Rodrigues *et al*., 2011), other cholinesterases may have been induced. Although all cholinesterase inhibitors

are thought to cause toxicity through this general mechanism, the biological responses may vary because different cholinesterase inhibitors may have different degrees of inhibition; i.e., the same inhibitor may produce varying responses among different species and tissues and can vary depending on the exposure pathways. Therefore, in monitoring studies the factors described previously must be taken into account (Jung et al., 2007). It is suggested that other cholinesterases (non-AChE) may interfere with compounds tested, and therefore the activity of AChE may be under-estimated.

Behavioral results and their relation to AChE activity

Behavioral endpoints have been shown to provide integrative measures of neurotoxicity. Thus, this study evaluated the role of neostigmine and pyridostigmine in the behavior of *Lepomis gibbosus*, by determining the % of time in black, % time in periphery and % time in motion. Several studies have tested the preference to light/dark in different species, which preferred the black compartment, probably seeking protection (Castro *et al.*, 2009; Maximino *et al.*, 2010). Maximino *et al*. (2010) developed a protocol to assess the light/dark preference. Castro et al. (2009) also postulated that the fish prefer the dark areas. Castro et al. (2009) also postulated that the fish prefer the dark areas and after exposure to different environmental pollutants, organisms have a greater indifference in the choice, ie, less choice and search for dark areas.

The work by Maximino *et al*. (2010) was the basis of the here adopted protocol for behavioral assessment. However, in preliminary tests (unpublished data), we found that most of the fish, after finding a protection zone (usually associated to the black compartment), remained motionless. As such, we evaluate the time spent in the dark compartment. Another important observation made during the preliminary tests, showed that most fish preferred areas close to the tank walls (periphery), regardless of being in the white or black compartment. Thus, we also considered quantifying the percentage of time in the periphery. Given that the enzyme AChE is the target of neostigmine and pyridostigmine, and that it is involved in the mechanisms of neuromuscular transmission, we also assessed the percentage of time during which the fish was actively swimming, in order to assess whether these drugs affected fish motion. Little *et al*. (1993) suggested counting the number of times the fish moved, for 2 minutes. However, we found interesting to assess the percentage of time during which the animal was in motion, because in our preliminary tests, most fish swam continuously for a period of time and then stopped. Being completely still, it was impossible to record any behavioral change; however, this drawback was overcome by artificially stimulating the fish to move, with a gentle touch with an external object (stage 2). The main purpose of performing stage 2 was to evaluate how the fish reacted after

stimulation. Thus, the determination of behavioral parameters in stage 2 allows us to assess whether the fish seeks new refuge and compare the behavioral responses obtained in stage 1.

In our study, the percentage of motion describes the percentage of the total record period that the fish spent moving, and this parameter has been evaluated in other studies directly (Ballesteros *et al.*, 2009) or indirectly (Correia *et al.*, 2007; Nunes *et al.*, 2008). In their study, Ballesteros *et al*. (2009) also quantified the motion percentage from *Jenynsia multidentata*, after exposure to 0.072 and 1.4 mg L^{-1} of endosulfan. In this study, swimming activity of individual (single) fish was recorded during 48 h, considering 10 min periods per hour. Correia *et al*. (2007) developed a study with *Sparus aurata* and each individual behavioral activity was categorized into three types: (i) swimming patterns (horizontal or vertical movements), (ii) lethargy, described as a non-locomotory activity by the absence of detectable body movements, and (iii) social patterns (social interaction). In our work, indirectly, we also evaluated lethargy, because we quantified the percentage of time spent in motion. Nunes *et al*. (2008) also quantified lethargy (normal animals exhibited continuous movement, and moved randomly inside the test recipient), after exposure to widely used pharmaceuticals and a detergent.

In our study, high individual variability of responses resulted in no significant differences among experimental treatments. However, a higher preference (greater than 50%) for the black compartment and the peripheral zone was found in individuals exposed to neostigmine (Figure 4) and pyridostigmine (Figure 5), in step 1. These results are consistent with those from studies of Castro *et al.* (2009) and Maximino *et al.* (2010), regarding the preference for black areas, as demonstrated by the fish. The stimulus forced the fish to move towards the opposite compartment. This may explain the decrease in the percentage attributed to the black compartment and to the periphery, relatively to the first stage. Data on % time in the periphery showed some apparent differences between fish exposed to neostigmine and pyridostigmine, noting a lower preference for the periphery of the fish exposed to pyridostigmine. These results may be associated with the fact that pyridostigmine have a more prolonged action than neostigmine, as described by previous studies (see previous section). Fish spent most of the time motionless, both for exposures for neostigmine (figure 4) and pyridostigmine (figure 5), for both stages 1 and 2. These results were already expected, since in our preliminary tests, the fish deterred themselves after finding a place that protects them.

Considering results from both behavior and AChE activity, it is interesting to note that fish exposed to neostigmine and pyridostigmine did not show changes in muscle AChE activity, except for fish exposed to 100 000 µg L^{-1} of pyridostigmine. The absence of significant changes in behavioral parameters analyzed may be related to the absence of significant changes in the activity of AChE in muscle. However, Barbier *et al*. (2009) describes that pyridostigmine treatment

administered in repeated stress conditions results in long-term perturbations of learning and social behavior. Our results suggest that the behavioral parameters analyzed in *L. gibbosus* cannot be regarded as a suitable marker to assess the effect of drugs such as neostigmine and pyridostigmine. In fact, data show that AChE was a more sensitive parameter than behavior alterations. This was an expected scenario, since observable effects at a low biological level are detectable at higher levels of organization later in time and at higher concentrations (Burton, 1991; Peakall, 1992), as a direct consequence of the integration of toxic effects along the scale of biological organization.

Several studies have proposed that the inhibition of AChE was associated with significant behavioral changes, since the activity of the nervous system is vital to normal behavior and muscular function. The loss of muscular control, due to inhibition of AChE, can cause multiple problems to fish, including loss of swimming control and stop of opercular motion. As the fish loses the ability to effectively move water across the gills, it faces a situation analogous to respiratory failure in mammals. This can lead to a reduced oxygenation of the blood, with the concomitant hypoxia-induced death (Ballesteros *et al.,* 2009). Despite of these important effects of AChE in muscle, brain AChE has been more studied than muscular. Arufe *et al.* (2007) refers that the inhibition of AChE is responsible for an impairment of neuronal and neuromuscular function and results in overstimulation of neurotransmission followed by depression or paralysis and eventual death. Based in previous assumptions would be expected significant changes in behavior.

Drug residues found in the aquatic environment usually occur as mixtures, not as single contaminants. So, when conducting toxicity studies, researchers should take into account the possible toxicological interactions (e.g., synergistic, additive, antagonistic) (Cleuvers, 2003). In the aquatic environment, pharmaceuticals are present as complex mixtures from many different classes. In addition, anticholinesterase drugs, such as neostigmine and pyridostigmine, and several other anticholinesterasic compounds can be found. Several studies have shown that AChE is inhibited by several compounds, including pesticides (Mora *et al.,* 1999; Fulton e Key, 2001; Binelli *et al.,* 2006; Feng *et al*., 2008; Ballesteros *et al*., 2009; Pereira *et al*., 2010; Tilton *et al*., 2011), heavy metals (Garcia *et al.,* 2000; Nunes *et al.,* 2003; Vieira *et al.,* 2009), mine waste (Castro *et al.,* 2004), pulp waste and detergents (Nunes *et al.,* 2005; Feng *et al.,* 2008; Li, 2008) and pharmaceuticals (Nunes *et al.,* 2006; Gonçalves *et al.,* 2010). Thus, pharmaceutical compounds represent a considerable part of active environmental contaminants that are persistent. In this study, each compound tested alone may have elicited little effect, but when acting together they may pose a hazard, which may be underestimated by focusing on individual compounds alone, as referred Fent *et al*. (2006). It is suggested therefore that the combined toxicity of several anticholinesterasic compounds (similar modes of action) in the environment and its effects on exposed organisms is also due in part to the drugs that are present, including neostigmine and pyridostigmine. Thus the

analysis of a suite of pharmaceuticals in a complex mixture would provide a more realistic situation in the environment (Quinn *et al*., 2009). So, considering the situation in the field with mostly very low concentrations of pharmaceuticals, we must assume that generally chronic effects are more likely than acute effects, and there may be other species that are more sensitive to these contaminants. In addition, more data on the chronic effects in fish are essential for estimating the environmental risk of pharmaceuticals.

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Capítulo IV

Considerações finais

A poluição ambiental decorrente das actividades antropogénicas exige o desenvolvimento de novas metodologias para avaliação, compreensão, prevenção e remediação dos efeitos nefastos para os sistemas biológicos. Actualmente os problemas ambientais são inúmeros, complexos e caracterizam-se essencialmente pela identificação e avaliação da toxicidade de xenobióticos, entre os quais os compostos anticolinesterásicos. Este impacto suscita grande preocupação científica em relação às espécies não-alvo existentes, nomeadamente nos ecossistemas aquáticos (e.g. perca-sol, *Lepomis gibbosus*). Tradicionalmente, os compostos anticolinesterásicos que suscitaram maior atenção foram os pesticidas organofosforados e estéres do ácido carbâmico, cujo efeito decorre da inibição selectiva das colinesterases. No entanto, um grupo específico de agentes químicos, os fármacos anticolinesterásicos, foram igualmente encontrados no compartimento aquático, levantando questões toxicológicas semelhantes às estudadas para os pesticidas anticolinesterásicos.

Vários são os parâmetros biológicos que podem ser alterados como consequência da interacção entre os agentes químicos e o organismo. No entanto, a determinação quantitativa desses parâmetros, usados como indicadores biológicos ou biomarcadores, só é possível se existir correlação com a intensidade da exposição e/ou efeito biológico da substância. Desta forma, a utilização de respostas induzidas por xenobióticos em componentes moleculares, celulares ou bioquímicos, apresentam grande potencial como ferramenta de avaliação de sinais precoces de alteração ao nível sub-individual, individual e até, possivelmente, ao nível da população ou da comunidade. A monitorização de um biomarcador específico, inibição da acetilcolinesterase (AChE) em peixes, tem sido amplamente utilizada em estudos ecotoxicológicos como um indicador de exposição a poluentes neurotóxicos. No entanto, não é de descartar a possibilidade da inibição das colinesterases poder ocorrer por influência de agentes tão diversos como fármacos de utilização humana.

Neste trabalho, foram obtidos dados ecotoxicológicos sobre os efeitos de alguns compostos anticolinesterásicos nomeadamente pesticida organofosforado (clorfenvinfos) e fármacos antimiasténicos (neostigmina e piridostigmina), bem como um detergente (SDS) em *L. gibbosus*.Através desta informação é possível agir, com maior conhecimento, em futuras avaliações de risco e/ou monitorização ambiental destes compostos. No Capitulo II, deste trabalho verificou-se que a acetilcolinesterase é a forma colinesterásica predominante na cabeça e no músculo de *L. gibbosus*. AChE está presente num grande número de organismos, principalmente no sistema nervoso. Através de exposições *in vivo* e *in vitro* observou-se que as colinesterases de *L. gibbosus* sofreram inibições significativas após exposição ao insecticida clorfenvinfos. Homogeneizados de cabeça e músculo mostraram capacidades de resposta e sensibilidades

semelhantes com este pesticida. Estes resultados confirmam o facto de a AChE ser um biomarcador fulcral para avaliar eventuais contaminações por pesticidas organofosforados. Contudo, para o detergente SDS, *L. gibbosus* revelou ausência de efeitos inibitórios na AChE, contrariamente ao verificado por alguns estudos anteriores.

No Capítulo III, a AChE foi utilizada como biomarcador para avaliar se a sua inibição poderá ou não implicar alterações comportamentais significativas em *L. gibbosus*, quando exposta a fármacos anticolinesterásicos (neostigmina e piridostigmina). Os resultados revelaram efeitos inibitórios na actividade da AChE mais acentuados para a piridostigmina do que para a neostigmina. Contudo, os ensaios comportamentais apresentaram elevada variabilidade, revelando-se assim incapazes de detectar alterações significativas nos organismos expostos, o que poderá questionar o uso desta ferramenta para avaliação de compostos anticolinesterásicos.

Os resultados obtidos neste estudo sugerem que a espécie *L. gibbosus* evidencia sensibilidade a pesticidas e fármacos anticolinesterásicos, sendo um potencial candidato a espécie bio-indicadora para a monitorização e contaminação de ambientes aquáticos. A inibição da AChE foi significativa para o clorfenvinfos e para fármacos. Os intervalos de concentrações dos xenobióticos adoptados nas exposições laboratoriais podem ser considerados, ecotoxicologicamente relevantes, atendendo às concentrações que podem ser encontradas no ambiente em ecossistemas contaminados. Relativamente, à avaliação de efeitos de fármacos anticolinesterásicos em peixes, este trabalho apresenta-se como um dos pioneiros, uma vez que observamos que estes induzem inibição da AChE.

Dado que os organismos no meio ambiente estão expostos a misturas de contaminantes, incluindo uma variedade de compostos anticolinesterásicos, seria relevante avaliar o efeito combinado destes compostos anticolinesterásicos, estudando eventuais sinergismos e/ou antagonismos. A toxicidade de compostos avaliada por exposição individual poderá ser sub ou sobre-estimada, se a extrapolar-mos para uma situação ambiental.

Contudo a necessidade de perceber prematuramente o que poderá acontecer numa escala de tempo mais alargada permite prevenir e/ou evitar efeitos biológicos mais acentuados na biota. O aumento do tempo de exposição deverá ser um critério a adoptar em trabalhos futuros, assim como a inclusão da análise de outros tecidos, tais como as brânquias, fígado, tubo digestivo e do estudo integrado de outros biomarcadores (stress oxidativo, biotransformação, peroxidação lípidica, etc).